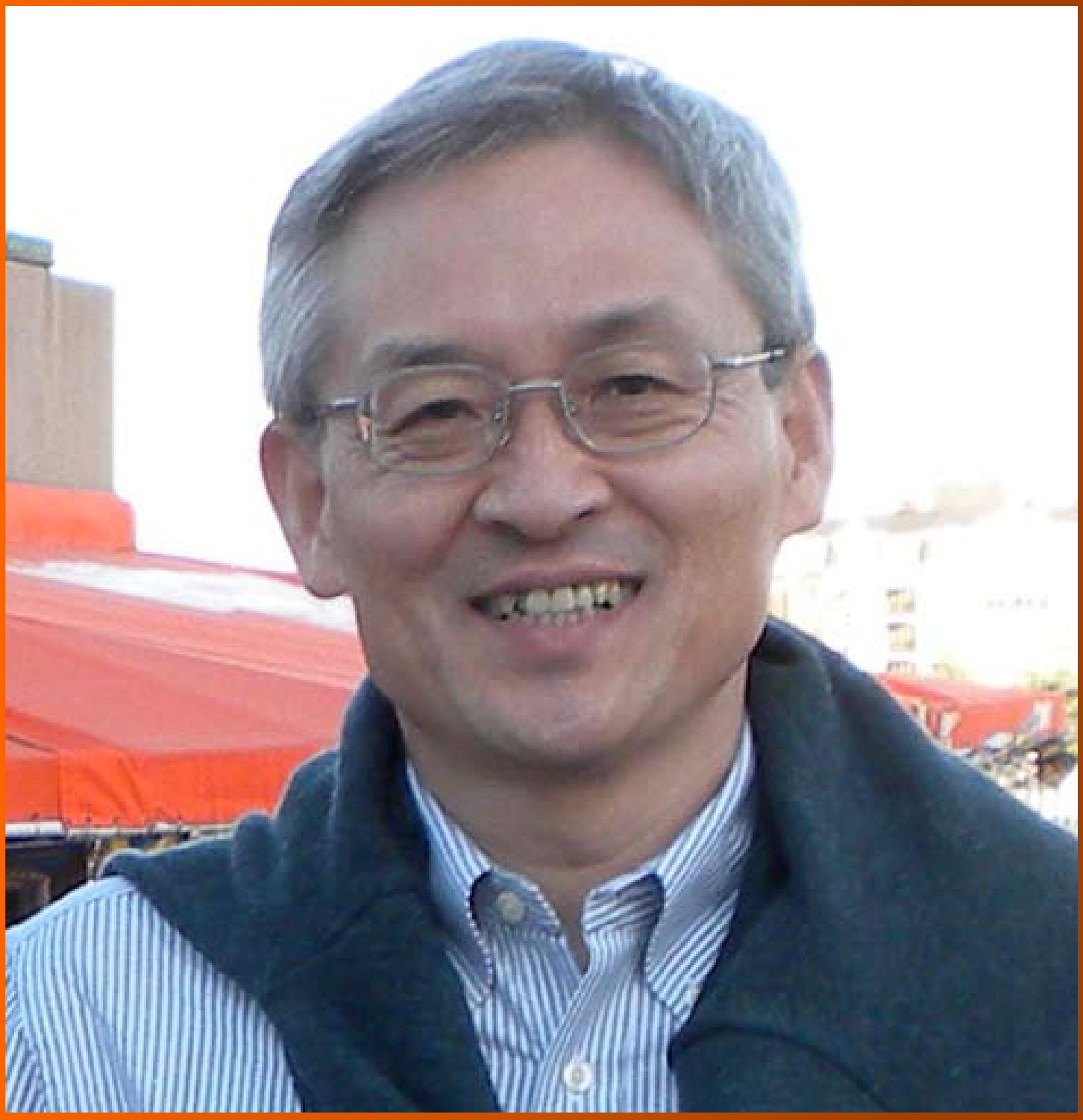
ISSN 2219-2824 (online)

World Journal of *Immunology*

World J Immunol 2014 July 27; 4(2): 42-129



Published by Baishideng Publishing Group Inc

WJI

World Journal of Immunology

A peer-reviewed, online, open-access journal of immunology

Editorial Board

2011-2015

The *World Journal of Immunology* Editorial Board consists of 247 members, representing a team of worldwide experts in immunology. They are from 33 countries, including Argentina (3), Australia (7), Austria (1), Belgium (1), Brazil (1), Canada (12), China (22), Czech Republic (1), Finland (1), France (14), Germany (8), Greece (2), Hungary (1), India (9), Ireland (1), Israel (6), Italy (16), Japan (9), Lebanon (1), Mexico (2), Netherlands (6), Norway (2), Peru (1), Portugal (2), Russia (3), Singapore (2), Slovenia (2), South Korea (6), Spain (7), Sweden (4), Switzerland (1), United Kingdom (14), and United States (79).

Martin Leverkus, *Mannheim*
Michael Linnebacher, *Rostock*
Jan Hendrik Niess, *ULM/Donau*
Enno Schmidt, *Luebeck*
Robert Weissert, *Regensburg*

Greece

Giorgos T Bamias, *Athens*
Clio P Mavragani, *Athens*

Hungary

Viktor Müller, *Budapest*

India

Arbind Acharya, *Varanasi*
Atmaram Hari Bandivdekar, *Mumbai*
Tapas Biswas, *Kolkata*
Keya Chaudhuri, *Kolkata*
Deepak Kaul, *Chandigarh*
Debashis Mitra, *Pune*
Praveen Rishi, *Chandigarh*
Shyam Sundar, *Varanasi*
Mohan R Wani, *Pune*

Ireland

Anne Fiona McGettrick, *Dublin*

Israel

Jacob George, *Rehovot*
Noah Isakov, *Beer Sheva*
Aaron Lerner, *Haifa*
David Naor, *Jerusalem*
Michal Schwartz, *Rehovot*
Elias Toubi, *Haifa*

Italy

Roberto Biassoni, *Genoa*
Francesco Indiveri, *Genoa*
Pietro Invernizzi, *Rozzano*
Lucia Lopalco, *Milan*
Angelo Martino, *Rome*
Ivano Mezzaroma, *Rome*
Antonella d'Arminio Monforte, *Milan*
Giulio Cesare Passàli, *Siena*
Carlo Perricone, *Rome*
Alessandro Poggi, *Genoa*
Antonella Prisco, *Naples*
Frncesco Recchia, *Avezzano*
Carlo Riccardi, *Perugia*
Domenico Sansonno, *Bari*
Margherita Sisto, *Bari*
Rosalinda Sorrentino, *Salerno-Fisciano*

Japan

Miyuki Azuma, *Tokyo*
Kozo Fujisaki, *Kagoshima*
Shigetsugu Hatakeyama, *Sapporo*
Kenji Kabashima, *Kyoto*
Ryuji Kubota, *Kagoshima*
Osam Mazda, *Kyoto*
Toshi Nagata, *Hamamatsu*
Toshimitsu Uede, *Sapporo*
Hisanori Umehara, *Kahoku-gun*

Lebanon

Nayef E Saadé, *Beirut*

Mexico

Carlos Rosales, *Mexico City*
Gilberto Vargas-Alarcón, *Mexico City*

Netherlands

Marianne Boes, *Utrecht*
Niels Bovenschen, *Utrecht*
Wouter J de Jonge, *Amsterdam*
J Wouter Jukema, *Leiden*
Frank A Redegeld, *Utrecht*
Ruurd Torensma, *Nijmegen*

Norway

Guanglin Cui, *Tromso*
Azzam A Maghazachi, *Oslo*

Peru

Salim Mohanna, *Lima*

Portugal

Alexandre M Carmo, *Porto*
Nuno M de Oliveira Lages Alves, *Porto*

Russia

Alexander S Apt, *Moscow*
Georgy A Nevinsky, *Novosibirsk*
Alexander B Poletaev, *Moscow*

Singapore

Jeak Ling Ding, *Singapore*
Alessandra Mortellaro, *Singapore*

Slovenia

Blaz Rozman, *Ljubljana*
Snezna Sodin-Semrl, *Ljubljana*

South Korea

Sin-Hyeog Im, *Gwangju*
Mi-Yeon Kim, *Seoul*
Hyung-Joo Kwon, *Chuncheon Gangwon-do*
Won-Ha Lee, *Daegu*
Cheol-Heui Yun, *Seoul*

Spain

Santos Mañes Brotón, *Madrid*
Joan Claria, *Barcelona*
Oscar J Cordero, *Santiago de Compostela*
Victoriano Mulero, *Murcia*
Mª Angeles Muñoz-Fernández, *Madrid*
Yolanda Revilla Novella, *Madrid*
Annabel F Valledor, *Barcelona*

Sweden

Francesco Dieli, *Stockholm*
Levitskaya Jelena, *Stockholm*
Stefan Karlsson, *Lund*
Zou Xiang, *Gothenburg*

Switzerland

Silvia Monticelli, *Bellinzona*

United Kingdom

Peter Barnes, *London*
Nicola Cirillo, *Bristol*
Rossen Mintchev Donev, *Swansea*
Eyad Elkord, *Manchester*
Fang-Ping Huang, *London*
John Maher, *London*
Claudio Nicoletti, *Norwich*
Dipak P Ramji, *Cardiff*
Cordula Margaret Stover, *Leicester*
Vadim V Sumbayev, *Chatham Maritime*
Ying Sun, *London*
Ping Wang, *London*
Xiao-Qing Wei, *Cardiff*
Heather M Wilson, *Aberdeen*

United States

Edward Abraham, *Birmingham*
Jessy J Alexander, *Chicago*
Robert J Amato, *Houston*
Hossam M Ashour, *Detroit*
Paul Ashwood, *Sacramento*
Sami L Bahna, *Shreveport*
Richard B Bankert, *Buffalo*
Igor M Belyakov, *Frederick*
Lauren Claire Berkow, *Baltimore*
Michael Borchers, *Cincinnati*
John J Bright, *Indianapolis*
Stuart K Calderwood, *Boston*
Christopher Chang, *Philadelphia*
Arvind Chhabra, *Farmington*
Lukasz K Chlewicki, *Chicago*
Yingzi Cong, *Galveston*
William Cruikshank, *Boston*
Peter Demant, *Buffalo*
Lauri J Diehl, *South San Francisco*

WJI

World Journal of Immunology

Contents

Four-monthly Volume 4 Number 2 July 27, 2014

REVIEW

42 Lysosomal acid lipase is critical for myeloid-derived suppressive cell differentiation, development, and homeostasis
Yan C, Du H

52 Gut immune response in the presence of hepatitis C virus infection
Hetta HF, Mehta MJ, Shata MTM

63 CD28/CTLA-4/B7 and CD40/CD40L costimulation and activation of regulatory T cells
Vogel IT, Van Gool SW, Ceuppens JL

78 Immunopathogenesis of reactive arthritis: Role of the cytokines
Eliçabe RJ, Di Genaro MS

88 Update on pythiosis immunobiology and immunotherapy
Loreto ÉS, Tondolo JSM, Zanette RA, Alves SH, Santurio JM

98 GM3-containing nanoparticles in immunosuppressed hosts: Effect on myeloid-derived suppressor cells
Fernández A, Oliver L, Alvarez R, Fernández LE, Mesa C

107 Role of host immune responses in sequence variability of HIV-1 Vpu
Hasan Z, Kamori D, Ueno T

116 Targeting TLR4/MAPKs signaling pathway: A better option for therapeutic inhibition of atherosclerosis
Plakkal Ayyappan J, Abraham A

MINIREVIEWS

122 Biologic response modifiers in retinal vasculitis
Saxena S, Srivastav K

Contents

World Journal of Immunology
Volume 4 Number 2 July 27, 2014

APPENDIX I-V Instructions to authors

ABOUT COVER Editorial Board Member of *World Journal of Immunology*, Hyung-Joo Kwon, PhD, Professor, Department of Microbiology, Hallym University College of Medicine, 39 Hallymdaehak-gil, Chuncheon Gangwon-do 200-702, South Korea

AIM AND SCOPE

World Journal of Immunology (*World J Immunol*, *WJI*, online ISSN 2219-2824, DOI: 10.5411) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJI covers a wide range of subjects including: (1) autoimmune diseases such as type 1 diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, thyroiditis, myasthenia gravis, in both humans and animal models of disease, with an interest on aspects including the etiology, pathogenesis, mechanisms of disease induction, maintenance and progression; (2) tumor immunology including immunosurveillance, immunoediting and immunotherapies in animal models and in humans; (3) clinical immunology in humans and animal models including mechanisms of disease, regulation and therapy and immunodeficiencies; (4) innate immunity including cell subsets, receptors and soluble mediators, complement and inflammation; (5) adaptive immune mechanisms and cells including soluble mediators and antibodies; (6) immune cell development, differentiation, maturation; (7) control mechanisms for immune cells including immune tolerance and apoptosis; (8) immune cell interactions and immune cell receptors; (9) immunological methods and techniques; (10) immune cell activation including cell signaling pathways, biochemical and pharmacologic modulation studies; (11) infection; (12) different modalities of vaccination including gene therapy; (13) hypersensitivity and allergy; (14) transplantation.

We encourage authors to submit their manuscripts to *WJI*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

INDEXING/ABSTRACTING

World Journal of Immunology is now indexed in Digital Object Identifier.

FLYLEAF I-III Editorial Board

NAME OF JOURNAL
World Journal of Immunology

ISSN
ISSN 2219-2824 (online)

LAUNCH DATE
December 27, 2011

FREQUENCY
Four-monthly

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
July 27, 2014

World Journal of Immunology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5411/wji.v4.i2.42

World J Immunol 2014 July 27; 4(2): 42-51
ISSN 2219-2824 (online)

REVIEW

Lysosomal acid lipase is critical for myeloid-derived suppressive cell differentiation, development, and homeostasis

Cong Yan, Hong Du

Cong Yan, Center for Immunobiology, Indiana University School of Medicine, Indianapolis, IN 46202, United States
Cong Yan, Hong Du, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN 46202, United States
Cong Yan, Hong Du, IU Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202, United States
Author contributions: Yan C and Du H wrote and edited the review.
Supported by National Institutes of Health, No.CA138759, CA152099, to Yan C; HL087001, to Du H, and HL-061803 and HL-067862 to Yan C and Du H
Correspondence to: Dr. Cong Yan, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, 975 W Walnut Street, IB424G, Indianapolis, IN 46202, United States. coyan@iupui.edu
Telephone: +1-317-2786005 Fax: +1-317-2788198
Received: February 26, 2014 Revised: April 2, 2014
Accepted: June 18, 2014
Published online: July 27, 2014

Abstract

Lysosomal acid lipase (LAL) cleaves cholesteryl esters (CE) and triglycerides (TG) to generate cholesterol and free fatty acid in lysosomes of cells. The downstream metabolic products of fatty acids are ligands for activation of peroxisome proliferator-activated receptor gamma (PPARγ). Accumulation of CEs and TGs is resulted from lack of functional LAL in lysosomes of cells, especially in myeloid cells. One characteristic phenotype in LAL knock-out (*lal-/-*) mice is systemic elevation of myeloid-derived suppressive cells (MDSCs). MDSCs infiltrate into multiple distal organs, alter T cell development, and suppress T cell proliferation and lymphokine production in *lal-/-* mice, which lead to severe pathogeneses in multiple organs. The gene transcriptional profile analysis in MDSCs from the bone marrow has identified multiple defects responsible for MDSCs malformation and malfunction in *lal-/-* mice, including G protein signaling, cell cycles, glycolysis metabolism, mitochondrial bioenergetics, mTOR pathway etc. In a separate gene transcriptional profile analysis in the lung of *lal-/-* mice, matrix metalloproteinase 12 (MMP12) and apoptosis inhibitor 6 (Api6) are highly overexpressed due to lack of ligand synthesis for PPARγ. PPARγ negatively regulates MMP12 and Api6. Blocking the PPAR signaling by overexpression of a dominant negative PPARγ (dnPPARγ) form, or overexpressing MMP12 or Api6 in myeloid or lung epithelial cells in inducible transgenic mouse models results in elevated MDSCs and inflammation-induced tumorigenesis. These studies demonstrate that LAL and its downstream effectors are critical for MDSCs development, differentiation and malfunction.

Key words: Lysosomal acid lipase; Myeloid-derived suppressor cells; Immunosuppression; Myeloid-derived suppressive cell development; Hematopoiesis

Core tip: Neutral lipid metabolism is essential for myeloid cell proliferation and differentiation. This review summarizes the most recent discoveries that lysosomal acid lipase (LAL), an enzyme hydrolysing cholesteryl esters and triglycerides in lysosomes, plays a critical role in myeloid-derived suppressive cells (MDSCs) development, differentiation, and immune suppressive function. Both LAL knock-out and myeloid specific rescue of LAL knock-out mice are used in the studies. Doxycycline-inducible bitransgenic mouse models of LAL downstream genes are also generated to study MDSCs malformation and malfunction. The molecular pathways/mechanisms to connect LAL and MDSCs are characterized by microarray analyses of gene transcriptional profiles.

Yan C, Du H. Lysosomal acid lipase is critical for myeloid-derived suppressive cell differentiation, development, and homeostasis. *World J Immunol* 2014; 4(2): 42-51 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i2/42.htm DOI:

http://dx.doi.org/10.5411/wji.v4.i2.42

HISTORY OF LYSOSOMAL ACID LIPASE

Lysosomal acid lipase (LAL) cleaves cholesteryl esters (CE) and triglycerides (TG) in cell lysosomes. Mutation in the LAL gene results in Wolman disease (WD) of early infantile onset, and cholesteryl ester storage disease (CESD) of late onset. WD was first described by Dr. Wolman[1] in 1956 as severe malnutrition, hepatosplenomegaly, calcified adrenal glands, and death of children within the first few months of life. Affected WD infants display massive accumulations of CE and TG in the lysosomes of hepatocytes and Kupffer cells, as well as in macrophages throughout the viscera, which lead to liver failure, severe hepatosplenomegaly, steatorrhea, pulmonary fibrosis[2,3], and adrenal calcification and insufficiency[4,5]. Lipid engorged macrophages in intestinal villi lead to severe malabsorption and cachexia[2,4]. The average life span of WD is 3.5 mo[6]. CESD was initially described by Fredrickson, Schiff, Langeron, and Infante and their colleagues in 1967[7-10] and named by Partin and Schubert based on phenotype that exhibited hepatomegaly with increased hepatic levels of cholesteryl esters in 1969. CESD can be a more indolent progressive disease, which shows microvesicular steatosis leading to fibrosis and cirrhosis in the liver, increases atherosclerosis and premature demise[11-13]. Wolman disease and CESD result from allelic mutations at the LAL locus on human chromosome 10q23.2-q23.3 and are autosomal recessive traits. The gene spans 45 kb, has 10 exons, and contains no unusual structures, except for a large intron 3. The *LIPA* mutations found in Wolman disease include deletions and insertions that lead to premature stop codons and the consequent loss of LAL protein and activity[14]. The mutations found in CESD are usually missense mutations, either heteroallelic or homoallelic with another mutant *LIPA* gene[14].

Recently, some evidence started to emerge, showing altered mononuclear phagocyte differentiation [increased $CD14^+CD16^+$ and $CD14^+CD33^+$ cells, subsets of human myeloid-derived suppressive cells, or myeloid-derived suppressive cells (MDSCs)] in humans that were heterozygote carriers of LAL mutations[15]. Furthermore, patients with mutations in the *LAL* gene have been reported to be associated with carcinogenesis[16]. These clinical observations support the extensive characterization in animal models as described below.

LAL PROPERTIES

LAL is a key player in the modulation of cholesterol metabolism in all cells. On the surface membranes of various cells, there are multiple receptors that can deliver LDL-bound cholesteryl esters/triglycerides to lysosomes, but LAL is the only lipase in the lysosomes that hydrolyzes cholesteryl esters and triglycerides. Once cleaved by LAL, the free cholesterol and fatty acids enter the cytosol from lysosome. In LAL deficiency, cholesteryl esters and triglycerides cannot be cleaved; therefore, free cholesterol and fatty acids cannot leave the lysosome[17,18]. Cells sense this as an intracellular (cytosolic) cholesterol deficiency, and the cholesterol biosynthetic pathway is up-regulated to compensate.

Synthesized in the rough endoplasmic reticulum, LAL is a typical soluble lysosomal hydrolase, which is co-translationally glycosylated when it emerges into the endoplasmic reticulum lumen[18,19]. Following the removal of the leader sequence (21 amino acids), LAL is decorated with oligosaccharides that are remodeled during transit through the Golgi apparatus. The N-linked oligosaccharides are remodeled from high mannosyl to complex forms, with a mannose 6-phosphate being added, which serves as the lysosomal sorting targeting signal. The mannose 6-phosphate receptor system is used to deliver the newly synthesized LAL to the lysosome. LAL is not known to require cofactors for optimal hydrolysis, and it functions as a monomer. Unmodified mature protein (378 amino acids) has a predicted molecular weight approximately 42.5 kDa. Different molecular weights have been reported for purified human LAL[20-24]. Occupancy of the LAL N-glycosylation is essential for enzyme stability, *i.e.*, protection from rapid degradation[25].

LAL has significant similarity to other acidic lipases, for example, lingual lipase and gastric lipases that cleave similar substrates in the stomach. However, LAL is distinct from other lipases, including hormone-sensitive lipase, pancreatic lysophospholipid lipase, lecithin cholesterol acyl transferase, lipoprotein lipase, hepatic lipase, and pancreatic lipase[26]. All such lipases share a motif, Gly-X-Ser-X-Gly, that is an essential pentapeptide in the active site[27,28]. This pentapeptide occurs twice in LAL at serine 99 and serine 153, and specific mutation of serine 153 identified this residue as important to catalytic activity[23]. Like other lipases, LAL also has a catalytic triad of Ser_{153}, Asp_{423} and His_{353}[27].

GENE KNOCK-OUT PHENOTYPES AND MDSCS IN MICE

A Lipa knock-out mouse (*lal-/-*) has been created to understand the functional roles of LAL in disease pathophysiology, lipid metabolism, and therapeutic approaches[29,30]. The *lal-/-* phenotype resembles human CESD. It' s histopathologic and biochemical phenotypes are similar to human WD. The *lal-/-* mice are normal appearing at birth, but develop liver enlargement by 4 wk and have a grossly enlarged abdomen with hepatosplenomegaly, lymph node enlargement, and intestinal villus infiltration by foamy macrophages by 16 wk. Massive accumulation of CE and TG and macrophage storage develops in these and other organs[29,31-34]. Enzyme therapy has been studied in this model using human recombinant LAL (rhLAL) produced in several different eukaryotic systems[24,35,36]. These studies clearly show the potential for correction of

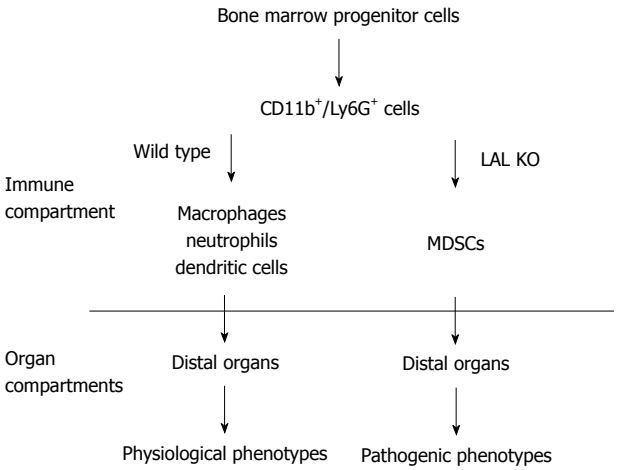


Figure 1 The functional role of Lysosomal acid lipase in myeloid lineage cells. In the wild type mice, the CD11b$^+$Ly6G$^+$ cells are myeloid lineage precursors for monocytes/macrophages, neutrophils, and dendritic cells, which participate in the normal physiological functions of the distal organs (*e.g.*, lung, liver, *etc.*), such as clearance of invading pathogens. The lysosomal acid lipase (LAL) activity is essential for normal myeloid lineage cell development, differentiation and function. LAL deficiency leads to neutral lipid accumulation in myeloid cells and blocks CD11b$^+$Ly6G$^+$ cells from further differentiation into mature myeloid lineage cells. The accumulated CD11b$^+$Ly6G$^+$ cells possess various malfunctions that participate in the pathogenic conditions in the residing organs. MDSCs: Myeloid-derived suppressive cells.

the manifestations if enzyme therapy is begun early in the course of the disease[36,37].

Many phenotypes of seemingly unrelated diseases in various organs co-exist in *lal*-/- mice. Therefore, these diseases must share common cellular and molecular mechanisms that link these pathological processes. Extensive characterization of *lal*-/- mice shows that elevation of systemic MDSCs is a major manifestation in association with most of the pathogenic conditions (*e.g.*, > 70% in the bone marrow and > 40% in the blood), suggesting that MDSCs play a central role in mediating LAL deficiency-induced pathogenic progression[29,31-34,36,38-41]. MDSCs was originally identified in tumor pathogenesis[42]. Recent studied have linked this cell population to many other chronic inflammatory diseases[43-50]. MDSCs are a mixture of myeloid cells that express CD11b and Gr-1 antigens in mice. In certain disease conditions (cancer), MDSCs are categorized into granulocytic (CD11b$^+$, Ly6G$^+$) and monocytic (CD11b$^+$Ly6C$^+$) MDSC[51]. Interestingly, most gated *lal*-/- CD11b$^+$ cells show Ly6C$^+$ and Ly6G$^+$ double positive, making them CD11b$^+$Ly6C$^+$ Ly6G$^+$ cells[34]. Normally, healthy immature myeloid lineage cells differentiate into dendritic cells (DCs), macrophages, or granulocytes in response to environmental changes. However, this process is blocked by LAL deficiency, leading to accumulation and expansion of MDSCs with immune suppressive function[51-53]. This is similar to what has been observed in the tumor environment[54]. It is conceivable that through paracrine and autocrine mechanisms, abnormally elevated MDSCs generate and secrete growth factors, chemokines and cytokines to influence cell differentiation, cell proliferation, cell apoptosis and gene expression in residing organ tissues, contributing to the physiological progression of various diseases. Direct cell-cell contact by MDSCs and other cells through the juxtacrine mechanism also contributes to this pathogenic process.

The functional roles of LAL in myeloid cells have been specifically evaluated by creating a myeloid-specific doxycycline-inducible c-fms-rtTA/(tetO)$_7$-CMV-hLAL; *lal*-/- triple mouse model, in which human LAL is expressed in myeloid cells under the control of the 7.2 kb c-fms promoter/intron2 regulatory sequence in *lal*-/- mice[32,34,55]. The hLAL expression in myeloid lineage cells in this triple mouse model significantly reduced systemic MDSCs accumulation[34], reversed aberrant gene expression, and ameliorated pathogenic phenotypes[32]. Therefore, the normal biological function of myeloid cells requires normal neutral lipid metabolism (Figure 1).

MDSCS DIFFERENTIATION AND DEVELOPMENT

The myeloid linage cells undergo the sequentially differentiated and proliferated from hematopoietic stem cells through an increasingly lineage-restricted intermediate progenitors including common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs) in the bone marrow[56,57]. The number and frequency of primitive LSK (Lin$^-$/Sca-1$^+$/c-kit$^+$), CMP, and GMP populations in the bone marrow, systemic myeloid cell distribution are changed in *lal*-/- mice, leading to an expansion in CD11b$^+$/Gr-1$^+$ MDSCs[41]. Both increased proliferation and decreased apoptosis contribute to the expansion of MDSCs in *lal*-/- mice. *Lal*-/- mice also display increased numbers of high proliferative potential colony-forming cells (HPP-CFC), colony-forming unite of granulocyte and macrophage progenitor cells (CFU-GM), colony-forming unite of granulocytes (CFU-G) and colony-forming unite of macrophages (CFU-M) colonies from cultured bone marrow cells. When *lal*-/- bone marrow cells are transplanted into wild type mice, the donor CD11b$^+$/GR-1$^+$ myeloid cells in the blood, spleen, lung and bone marrow of recipient mice are increased, confirming that the MDSCs increase is primarily due to the intrinsic defect in myeloid lineage progenitor cells. In addition to the intrinsic progenitor problem, the environment in *lal*-/- mice also contributes to myeloid cell hyper-expansion, since the donor CD11b$^+$/GR-1$^+$ myeloid cell population in *lal*-/- recipient mice that are transplanted with wild type bone marrow cells is expanded. Therefore, the *lal*-/- environment does not normally support hematopoiesis. Deregulated bone marrow progenitor cell differentiation is a primary cause for expansion of *lal*-/- MDSCs, which is attributed to both cell-autonomous and environmental factors. Taken together, LAL expression in myeloid lineage cells is critical to maintain hematopoiesis and myelopoiesis. After MDSCs infiltration into distal organs, at least two mechanisms can explain how the cell-autonomous defect and environmental factors influence each other. Firstly, MDSCs and other regional

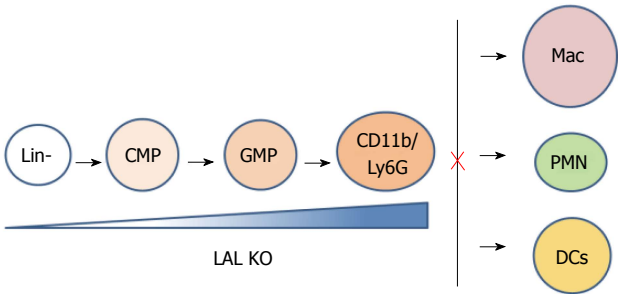


Figure 2 Lysosomal acid lipase is required for normal myeloid lineage cell development and differentiation. Lysosomal acid lipase (LAL) deficiency leads to increased myeloid-derived suppressive cells differentiation from Lin$^-$ progenitor cells in the bone barrow, and decreased differentiation to mature macrophages, neutrophils, and dendritic cells in other compartments. Lin$^-$: Lineage negative progenitor; CMP: Common myeloid progenitor; GMP: Granulocyte-macrophage progenitor; Mac: Macrophage; PMN: Polymorphonuclear cell, or neutrophil; DC: Dendritic cell.

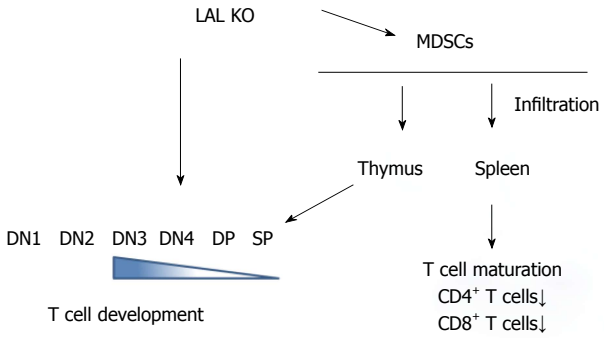


Figure 3 Lysosomal acid lipase is required for normal T cell development and differentiation. Lysosomal acid lipase (LAL) deficiency can cause the intrinsic defect in T cell development, starting at the double negative 3 (DN3) stage. In addition, myeloid-derived suppressive cells infiltrate into the thymus and spleen, resulting in blockage of normal T cell development, differentiation, and maturation. DN: CD4 and CD8 double negative; DP: CD4 and CD8 double positive; SP: CD4 or CD8 single positive.

cells in distal organs influence each other by the paracrine mechanism as both sides secrete cytokines and chemokines. Secondly, MDSCs and other cells can influence each other by direct contact (juxtacrine mechanism). Starting at the GMP stage, hLAL expression in myeloid cells reverses abnormal myeloid development in the bone marrow, and reduces systemic expansion of MDSCs in c-fms-rtTA/(tetO)$_7$-CMV-hLAL; *lal*-/- triple mice. In addition, differentiation from Lin$^-$ progenitor cells to CD11b$^+$GR-1$^+$ cells is abnormally increased in *lal*-/- mice (Figure 2). This further supports that the cell-autonomous effect of MDSCs expansion in *lal*-/- mice. Myeloid hLAL expression in c-fms-rtTA/(tetO)$_7$-CMV-hLAL; *lal*-/- triple mice successfully reverses this abnormality[32]. The environmental effects on MDSCs malformation are further supported by an observation that when the Stat3 pathway is overly activated in lung epithelial cells[58], secretion of Stat3-induced pro-inflammatory cytokines in epithelial cells reversed mature myeloid lineage cells to MDSCs[59].

MDSCS IMMUNOSUPPRESSION

In contrast to myeloid lineage cells, T cells are systemically decreased in *lal*-/- mice. *Lal*-/- T cells behave abnormally. In response to stimulation of anti-CD3 plus anti-CD28 antibodies, or phorbol-12-myristate-13-acetate (agonist to activate PKC) and ionomycin (calcium ionophore), there is severely diminished T cell proliferation, decreased CD69 expression, and decreased expression of T cell lymphokines. LAL deficiency does not drive effector T cells into either Th1 or Th2 status[33]. The thymus is the most important organ for T cell development, which is divided into different developmental stages that are marked by CD4$^-$CD8$^-$ double negative (DN) 1 to 4 stages, CD4$^+$CD8$^+$ double positive (DP) stage and CD4$^+$ or CD8$^+$ single positive (SP) stage. The earliest stage for thymocyte paucity appears at the DN4 (CD25$^-$CD44$^-$) stage in the *lal*-/- thymus. After this developmental point, thymocytes are declining at all stages, suggesting that the blockage of T cell development initially occurs at the DN3 to DN4 transition (Figure 3)[33]. Decrease of T cell development and maturation was also observed in *lal*-/- mice due to the defects in lymphoid progenitors in the bone marrow chimeras study. This notion has been supported by the bone marrow profile analysis, in which common lymphoid progenitor development is blocked in the bone marrow of *lal*-/- mice[33,41].

In addition to the above intrinsic defect, extensive analyses have revealed a second mechanism that contributes to systemic reduction of T cell populations. Strikingly, LAL deficiency dramatically increases MDSCs expansion and infiltration in the thymus and the spleen of *lal*-/- mice, leading to neutral lipid accumulation and abnormal organization of the thymus and spleen[33]. Infiltration of MDSCs in these important T cell organs affects T cell development, differentiation and maturation. Functional analyses have shown that MDSCs from *lal*-/- mice strongly inhibit proliferation and function of T cells (Figure 3)[34,40,41].

Direct connection between LAL in MDSCs and T cell abnormalities comes from the c-fms-rtTA/(tetO)$_7$-CMV-hLAL;*lal*-/- triple mouse study. MDSCs expansion and infiltration into the thymus and spleen are reduced in this mouse model. This leads to restoration of T cell proliferation in the spleen and normal T cell development in the thymus[34]. Stat3 and NFκB p65 signaling play a critical role in *lal*-/- MDSCs immune suppressive function[34]. The above observations are further proved by an MDSCs depletion study, in which anti-Gr-1 antibody treatment recovers T cell numbers in *lal*-/- mice[34]. *lal*-/- MDSCs also inhibits T cell lymphokine production, which is resulted from inactivation of the pZAP-70/Syk intracellular signaling, loss of expression of TCR ξ chain and CD69, a failure to respond to TCR stimulation[33]. These defects can also be reversed by myeloid hLAL expression[34]. Lastly, Treg cells inhibit CD4$^+$ T cell lymphokine production and proliferation[60]. LAL deficiency substantially increases CD4$^+$FoxP3$^+$ Treg cells in *lal*-/-mice[33].

GENE PROFILES IN LAL DEFICIENCY-INDUCED MDSCS

Since LAL controls homeostasis and development of MDSCs, which have profound pathogenic impact on various disease development, it is essential to identify the intrinsic defects that are involved in the MDSCs homeostasis and function for future targeting. In a comprehensive gene transcriptional profile study by Affymetrix GeneChip microarray analysis, multiple pathways have been revealed in *lal*-/- bone marrow MDSCs. Below are lists of some major (but not limited) changed pathways in *lal*-/- MDSCs.

Genes of G-protein superfamily

Expression changes of both large and small GTPases have been detected in *lal*-/- MDSCs, which have diverse functions in cells[61,62]. They include: (1) Rab GTPases, which control vesicle formation, receptor internalization, and trafficking to the nucleus, lysosome and plasma membrane. Rab GTPases regulate cellular proliferation, apoptosis and migration by integrating signaling pathways; (2) Rho GTPases, which organize actin cytoskeleton, cell adhesion and cell motility[63]; and (3) Ras GTPases mediate cell-cycle entry, cell growth, cell survival, cell growth and cellular metabolism by phosphorylating transcription factors through activation of the Raf/Mek/Erk pathway. Activation of Erk and p38 phosphorylation has been observed in *lal*-/- MDSCs[41].

Histone cluster genes and cell cycle genes

Cell cycle regulating genes are upregulated in *lal*-/- MDSCs. They include: (1) Histone-variants cluster genes, which favor the epigenetic microenvironment change to promote MDSCs expansion. Histone-variants exchange also contributes to formation of centromeric and telomeric chromatin during cell cycles. Indeed, G1/M phases of *lal*-/- MDSCs are increased in a cell cycle analysis[64]; (2) Cell cycle related genes[65], including Cdk1, Cdk2, Cdk5, Cdk9, and all Cdk regulatory cyclins (A, B, D, E-type), suggesting constitutive mitogenic signaling and defective responses to anti-mitogenic signals; and (3) Ubiquitination and proteasome enzymes/protein factors, which direct proteins to proteolysis within proteasome for recycling[66].

Metabolism and bioenergetics

Bioenergetic and metabolic genes are abnormally upregulated in *lal*-/- MDSCs, which control mitochondrial oxidative phosphorylation and energy (ATP production) for cellular activities. These include: (1) lactate dehydrogenase A and B, which produce large quantities of secreted lactate, suggesting that *lal*-/- MDSCs use an aerobic glycolysis; (2) nitric oxide/reactive oxygen species (ROS) production genes, glutathione peroxidase/glutathione reductase genes, and glucose 6-phosphate dehydrogenase gene, which are involved in production of ROS. The concentration of ROS is significantly increased in *lal*-/- MDSCs; (3) enzymes and proteins in glycolysis and citric acid cycles; and (4) respiratory chain proteins (NADH dehydrogenases, cytochrome proteins, ATPases and mitochondrial ribosomal proteins).

The mTOR pathway in LAL deficiency induced MDSCs

PI3K/thymoma viral proto-oncogene (AKT)/mammalian target of rapamycin (mTOR) is activated in *lal*-/- MDSCs[64]. mTOR is a lysosomal membrane-bound protein, which controls apoptosis, promotes influx of glucose and amino acids into the cells, stimulates ATP production[67], contributes to cell growth, cell cycle entry, cell survival, and cell motility[68,69]. Lack of the LAL activity changes lipid composition and dynamics on the lysosomal membrane that potentially influence endomembrane trafficking and stimulate the mTOR activity, which in turn coordinates the cellular metabolism[64,69,70]. It has been demonstrated that mTOR plays a critical role in modulating cellular immune functions[71,72], activation of the mTOR pathway contributes to *lal*-/- MDSCs production and function[40]. mTOR is the catalytic subunit of two distinctive complexes; mTOR complex 1 (mTORC1) and mTOR complex (mTORC2). mTORC1 contains unique regulatory associated proteins of mTOR (RAPTOR) while mTORC2 contains rapamycin-insensitive companion of mTOR (RICTOR)[67,72-75]. Inhibition of mTOR and associated proteins (Raptor, Rictor, and Akt1) corrects *lal*-/- MDSCs development, increased cell proliferation, decreased cellular apoptosis, and immune suppression in association with decreased ROS production, recovery from impairment of the mitochondrial membrane potential, increased ATP synthesis, and increased cell cycling. Potentially, the mTOR pathway can serve as a target to modulate the emergence of MDSCs in various pathophysiologic states where these cells play an immunosuppressive role (Figure 4).

The Stat3 and NFκB pathways

Although upregulation of Signal Transducer and Activator of Transcription (Stat) family members and NFκB family members are not detected by microarray analysis, phosphorylation of Stat3 and NFκB has been detected in expanded *lal*-/- MDSCs[34,41]. Activation of Stat3 directly leads to MDSCs expansion *in vivo*[58,59].

STUDY OF LAL DOWNSTREAM GENES

The gene profile study in the lung of *lal*-/- mice by Affymetrix GeneChip microarray analysis has also been performed. This is because the lung is a lipid rich organ and highly responsive to inflammation. Neutral lipids account for 10% of the composition of pulmonary surfactant that protects alveoli from collapse during respiratory cycles[76]. LAL deficiency results in massive myeloid cell infiltration, hyperplasia and emphysema in the *lal*-/- lung[32,39]. Comparison between the changed gene lists of bone marrow MDSCs and the whole lung by Affymetrix GeneChip microarray analyses reveals a few overlapping

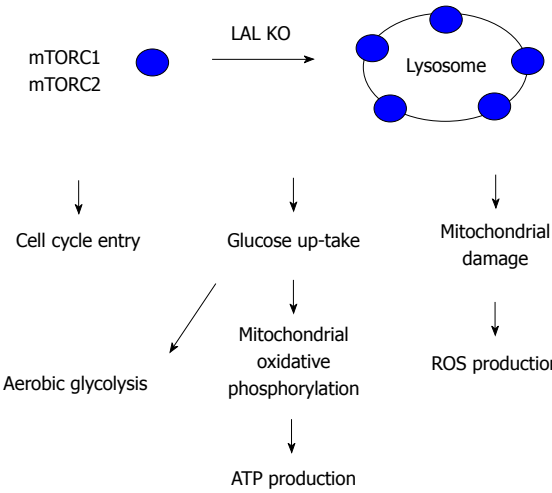


Figure 4 Lysosomal acid lipase deficiency induces overactivation of the mTOR pathway in myeloid-derived suppressive cells. Lysosomal acid lipase (LAL) is a lysosome-associated enzyme. LAL deficiency increases mTOR complexes anchoring on lysosomes and stimulates the mTOR1 activity to influence the cellular metabolism and proliferation of *lal*-/- myeloid-derived suppressive cells (MDSCs). These include an increased influx of glucose through aerobic glycolysis, an increased mitochondrial oxidative phosphorylation and ATP production, an impairment of the mitochondrial membrane potential in association with increased reactive oxygen species (ROS) production, and an increased cell cycle entry in *lal*-/- MDSCs.

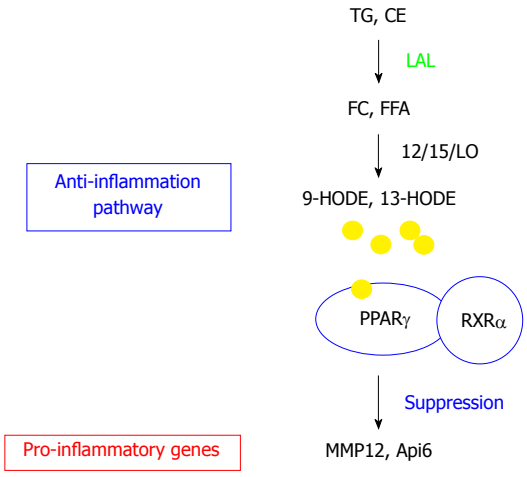


Figure 5 Lysosomal acid lipase and its downstream effector genes. Lysosomal acid lipase (LAL) cleaves cholesteryl esters (CE) and triglycerides (TG) to produce free cholesterol (FC) and fatty acids (FFA) in lysosomes of cells. The lipid derivatives (9-HODE, 13-HODE) of FFA serve as ligands for PPARγ in coupling with retinoid X receptor α (RXRα, which suppresses gene expression of a variety of pro-inflammatory cytokines. The LAL/PPARγ axis serves as an anti-inflammatory pathway. LAL deficiency blocks this metabolic pathway to provoke up-regulation of pro-inflammatory cytokines (*e.g.*, Api6, MMP12). TGF: Transforming growth factor beta; IL: Interleukin; MCP: Monocyte chemotactic protein; TNF: Tumor necrosis factor; NF: Nuclear factor.

genes. Therefore, LAL performs differential roles in different compartments. LAL exerts its biological effects through its downstream genes. In order to fully understand the LAL functions, it is necessary and essential to characterize its downstream genes. From the whole lung gene list, the two most up-regulated genes matrix metal proteinase 12 (MMP12) and apoptosis inhibitor 6 (Api6) are characterized extensively. The functional role of LAL downstream effector peroxisome proliferator-activated receptor gamma (PPARγ) has also been studied in depth. Figure 5 shows the relationship between LAL and its downstream effectors.

PPARγ

Involvement of the receptor network in the metabolic programming of myeloid lineage cells is essential to the innate immune system[77,78]. PPARγ is of high interest for several reasons. Firstly, the metabolites of LAL hydrolysis, 9-hydroxyoctadecanoic acids (9-HODE) or 13-HODE from linoleic acid, serve as ligands for PPARγ. Upon binding to the ligands, PPAR interacts with the retinoid X receptor (RXR) to form the PPARγ/RXR dimer on target genes. Secondly, PPARγ plays an important role in anti-inflammation of various tissues[77,79,80]. It has been shown that PPARγ agonists suppress gene expression of inflammatory cytokines[79]. In the *lal*-/- lung, these pro-inflammatory cytokines are up-regulated (Figure 5)[39]. Therefore, LAL deficiency causes inactivation of PPARγ by depleting ligand production. Using the lung as a model system, reintroduction of LAL downstream metabolic derivative 9-HODE (a natural occurring ligand for PPARγ) and a synthetic ligand compound ciglitazone for PPARγ improves the inflammatory status and pathogenesis in the *lal*-/- lung. Therefore, the ligands/PPARγ axis controls inflammation-triggered elevated gene expression and pathogenesis in the *lal*-/- mice[31].

To directly evaluate functional role of LAL downstream effector PPARγ in myeloid cells, dominant negative PPARγ (dnPPARγ) is overexpressed in a myeloid-specific c-fms-rtTA/(TetO)$_7$-CMV-dnPPARγ bitransgenic mouse model[81]. In this bitransgenic system, total numbers and frequencies of LK, LSK, CMP and GMP progenitor cells in the bone marrow are abnormally elevated. DnPPARγ overexpression leads to up-regulation of IL-1β, IL-6 and TNFα in the blood plasma. MDSCs from this bitransgenic mouse model inhibit the proliferation and lymphokine production of wild type $CD4^+$ T cells *in vitro*. Both $CD4^+$ and $CD8^+$ T cell populations are decreased in doxycycline-induced dnPPARγ expressed mice. Bone marrow transplantation reveals that a myeloid autonomous defect is responsible for MDSC expansion, immunosuppression and tumorigenesis in this myeloid-specifically expressed dnPPARγ bitransgenic mice. Multiple forms of carcinoma and sarcoma in various organs (the lung, liver, spleen and lymph nodes) are observed in this mouse model. Therefore, the LAL/hormonal ligands/PPARγ axis is critical to control inflammation and the induction of various tumors. Disruption of this pathway in myeloid cells, either by blocking ligand synthesis (as in *lal* -/- mice), or inhibition of PPARγ (as in c-fms-rtTA/(TetO)$_7$-CMV-dnPPARγ bitransgenic mice) can initiate up-regulation of inflammatory molecules which cause hematopoietic progenitors skewing towards myeloid lineage expansion to form MDSCs.

Matrix metalloproteinases12

Zinc-dependent MMPs act as modulators for inflammation and innate immunity by activating, deactivating or modifying the activities of signaling cytokines, chemokines and receptors through proteolytic and nonproteolytic functions[82-84]. Among MMPs, MMP12 is a 22-kDa secretory proteinase that is predominantly expressed in macrophages as previously reported[85]. MMP12 degrades extracellular matrix components, such as type IV collagen, fibronectin, laminin, gelatin, vitronectin, entactin, heparin, and chondroitin sulphates, to facilitate tissue remodeling[86]. The expression of MMP12 in macrophages is induced in the lung of cigarette smokers[87]. Inactivation of the MMP12 gene in knock-out mice demonstrates a critical role of MMP12 in smoking-induced chronic obstructive pulmonary disease (COPD)[88], a disease highly related to lung cancer. From clinical studies, MMP12 correlates with early cancer-related deaths in non-small cell lung cancer (NSCLC), especially with those associated with tobacco cigarette smoke exposure[89,90]. In the *lal*-/- lung, MMP-12 is the highest upregulated gene[31]. In the *lal*-/- lung, both macrophages and lung epithelial alveolar type Ⅱ (AT Ⅱ) cells are responsible for MMP-12 increase[31,91,92]. Both myeloid-specific and lung epithelia-specific MMP12 bitransgenic mouse models have been created to study the functional roles of this LAL/PPARγ downstream molecule.

In the myeloid-specific c-fms-rtTA/(TetO)$_7$-CMV-MMP12 bitransgenic mouse model, induction of MMP12 abnormally elevates numbers and frequencies of CMP and GMP populations in the bone marrow, similar to that observed in *lal*-/- mice. Addition of activated MMP12 is able to stimulate wild type Lin$^-$ progenitor cells to differentiate into the MDSC population, suggesting that MMP12 directly exerts its effect on hematopoietic progenitor cells. The MDSCs are systemically increased in multiple organs of MMP12 bitransgenic mice. MDSCs from MMP12-overexpred bitransgenic mice suppress T cell proliferation and function. MMP12 directly stimulates differentiation of CD11b$^+$Gr-1$^+$ cells from Lin$^-$ progenitor cells. In the lung, the concentration of IL-6 is increased, which aberrantly activates oncogenic Stat3 and increases expression of Stat3 downstream genes in epithelial tumor progenitor cells. As a result, spontaneous emphysema and lung adenocarcinoma are sequentially developed in MMP12-overexpressive bitransgenic mice, suggesting a critical role of MMP12 in the transition from emphysema to lung cancer.

In epithelial-specific CCSP-rtTA/(TetO)$_7$-CMV-MMP12 bitransgenic mice, MMP12 overexpression induces regional MDSCs infiltration and increases epithelial growth. Again, spontaneous emphysema and bronchioalveolar adenocarcinoma are developed sequentially. Importantly, MMP12 upregulation is highly associated with COPD and lung cancer in human patients. Together, these studies support that LAL/PPARγ downstream MMP12 plays a critical role in emphysema to lung cancer transition that is facilitated by inflammation. Clinically, it has been reported that there is a pathophysiological connection between emphysema/COPD and lung cancers[93,94].

Apoptosis inhibitor 6

Apoptosis inhibitor 6 (Api6) belongs to the macrophage scavenger receptor cysteine-rich domain superfamily (SRCR-SF)[95,96]. Api6 expression is the second highest induced gene in the *lal*-/- lung. Api6 is regulated by LAL metabolic derivatives (*e.g.*, 9-HODE) and PPARγ[31]. In a myeloid-specific c-fms-rtTA/(TetO)$_7$-CMV-Api6 bitransgenic mouse model, many phenotypes are similar to those observed in *lal*-/- mice. Overexpression of Api6 abnormally elevates MDSCs in the bone marrow, blood and lung with increased cell proliferation and decreased apoptotic activities. Api6 overexpression activates Stat3, Erk1/2 and p38 in myeloid lineage cells. Persistent inflammation in myeloid-specific Api6 bitransgenic mice causes lung adenocarcinoma[97].

Pathogenic overexpression of Api6 is also observed in *lal*-/- AT Ⅱ cells. In an epithelial-specific CCSP-rtTA/(TetO)$_7$-CMV-Api6 bitransgenic mice, Api6 overexpression in AT Ⅱ cells increases pro-inflammatory cytokine/chemokine levels in bronchoalveolar lavage fluid and serum, activates oncogenic signaling and inhibits apoptosis, promotes expansion of MDSCs in lung and blood but not in the bone marrow or spleen. Lung MDSCs from this bitransgenic mouse model suppress T cell proliferation and function, which results in occurrence of emphysema and adenocarcinoma.

CONCLUSION

MDSCs play vital roles in various inflammation-induced chronic diseases. Elimination or reduction of MDSCs populations can slow down disease formation and progression. It is important to identify the molecular pathways in order to effectively block MDSCs homeostasis and function. Extensive studies outlined in this review have shown that the role of LAL in controlling neutral lipid metabolism is a key player in MDSCs development, homeostasis and function, therefore, providing a new avenue to develop therapeutic or immunologic approaches for clinical application. Through studies of the LAL function, defective gene expression patterns have been mapped in *lal*-/- MDSCs. These provide novel targets for controlling MDSCs and associated diseases by designing small molecule inhibitors. Clinically, small molecule inhibitors for c-kit have been tested to target MDSCs[98]. Using the gene profile list from LAL deficiency-induced MDSCs, more small molecule inhibitors can and will be identified to inhibit MDSCs pathogenic functions in various disease conditions.

ACKNOWLEDGMENTS

The authors thank Miss Katlin Walls for proof-reading the manuscript.

REFERENCES

1 **Abramov A**, Schorr S, Wolman M. Generalized xanthomatosis with calcified adrenals. *AMA J Dis Child* 1956; **91**: 282-286 [PMID: 13301142]
2 **Boldrini R**, Devito R, Biselli R, Filocamo M, Bosman C. Wolman disease and cholesteryl ester storage disease diagnosed by histological and ultrastructural examination of intestinal and liver biopsy. *Pathol Res Pract* 2004; **200**: 231-240 [PMID: 15200275 DOI: 10.1016/j.prp.2003.11.001]
3 **Krivit W**, Peters C, Dusenbery K, Ben-Yoseph Y, Ramsay NK, Wagner JE, Anderson R. Wolman disease successfully treated by bone marrow transplantation. *Bone Marrow Transplant* 2000; **26**: 567-570 [PMID: 11019848 DOI: 10.1038/sj.bmt.1702557]
4 **Assmann G**, Seedorf U. Acid lipase deficiency: Wolman disease and cholesteryl ester storage disease. The Metabolic and Molecular Bases of Inherited Disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. 6 ed. New York: McGraw-Hill, 1995: 2563-2587
5 **Stein J**, Garty BZ, Dror Y, Fenig E, Zeigler M, Yaniv I. Successful treatment of Wolman disease by unrelated umbilical cord blood transplantation. *Eur J Pediatr* 2007; **166**: 663-666 [PMID: 17033804 DOI: 10.1007/s00431-006-0298-6]
6 **Reynolds T**. Cholesteryl ester storage disease: a rare and possibly treatable cause of premature vascular disease and cirrhosis. *J Clin Pathol* 2013; **66**: 918-923 [PMID: 23999269 DOI: 10.1136/jclinpath-2012-201302]
7 **Fredrickson DS**, Sloan HR, Ferrans VJ, Demosky SJ. Cholesteryl ester storage disease: a most unusual manifestation of deficiency of two lysosomal enzyme activities. *Trans Assoc Am Physicians* 1972; **85**: 109-119 [PMID: 4660005]
8 **Schiff L**, Schubert WK, McAdams AJ, Spiegel EL, O'Donnell JF. Hepatic cholesterol ester storage disease, a familial disorder. I. Clinical aspects. *Am J Med* 1968; **44**: 538-546 [PMID: 5642714 DOI: 10.1016/0002-9343(68)90054-5]
9 **Lageron A**, Caroli J, Stralin H, Barbier P. [Cholesterolic polycoria in adults. I. Clinical and histochemical study]. *Presse Med* 1967; **75**: 2785 [PMID: 5583022]
10 **Infante R**, Polonovski J, Caroli J. [Cholesterolic polycoria in adults. II. Biochemical study]. *Presse Med* 1967; **75**: 2829-2832 [PMID: 5583895]
11 **Beaudet AL**, Ferry GD, Nichols BL, Rosenberg HS. Cholesterol ester storage disease: clinical, biochemical, and pathological studies. *J Pediatr* 1977; **90**: 910-914 [PMID: 859064 DOI: 10.1016/S0022-3476(77)80557-X]
12 **Bernstein DL**, Hülkova H, Bialer MG, Desnick RJ. Cholesteryl ester storage disease: review of the findings in 135 reported patients with an underdiagnosed disease. *J Hepatol* 2013; **58**: 1230-1243 [PMID: 23485521 DOI: 10.1016/j.jhep.2013.02.014]
13 **Fouchier SW**, Defesche JC. Lysosomal acid lipase A and the hypercholesterolaemic phenotype. *Curr Opin Lipidol* 2013; **24**: 332-338 [PMID: 23652569 DOI: 10.1097/MOL.0b013e328361f6c6]
14 **Grabowski GA**, Du H. Lysosomal Acid Lipase Deficiencies: The Wolman Disease/Cholesteryl ester storage disease Spectrum. The Online Metabolic and Molecular Bases of inherited Disease (OMMBID). In: Valle D BA, Voglstein B, Kinzler KW, Antonarakis SE, editors. 9th ed. New York: McGraw-Hill, 2012
15 **Rothe G**, Stöhr J, Fehringer P, Gasche C, Schmitz G. Altered mononuclear phagocyte differentiation associated with genetic defects of the lysosomal acid lipase. *Atherosclerosis* 1997; **130**: 215-221 [PMID: 9126667 DOI: 10.1016/S0021-9150(97)06065-6]
16 **Elleder M**, Chlumská A, Hyánek J, Poupětová H, Ledvinová J, Maas S, Lohse P. Subclinical course of cholesteryl ester storage disease in an adult with hypercholesterolemia, accelerated atherosclerosis, and liver cancer. *J Hepatol* 2000; **32**: 528-534 [PMID: 10735626 DOI: 10.1016/S0168-8278(00)80407-9]
17 **Brown MS**, Sobhani MK, Brunschede GY, Goldstein JL. Restoration of a regulatory response to low density lipoprotein in acid lipase-deficient human fibroblasts. *J Biol Chem* 1976; **251**: 3277-3286 [PMID: 179993]
18 **Sando GN**, Ma GP, Lindsley KA, Wei YP. Intercellular transport of lysosomal acid lipase mediates lipoprotein cholesteryl ester metabolism in a human vascular endothelial cell-fibroblast coculture system. *Cell Regul* 1990; **1**: 661-674 [PMID: 2150334]
19 **Sando GN**, Henke VL. Recognition and receptor-mediated endocytosis of the lysosomal acid lipase secreted by cultured human fibroblasts. *J Lipid Res* 1982; **23**: 114-123 [PMID: 7057100]
20 **Sando GN**, Rosenbaum LM. Human lysosomal acid lipase/cholesteryl ester hydrolase. Purification and properties of the form secreted by fibroblasts in microcarrier culture. *J Biol Chem* 1985; **260**: 15186-15193 [PMID: 4066668]
21 **Ameis D**, Merkel M, Eckerskorn C, Greten H. Purification, characterization and molecular cloning of human hepatic lysosomal acid lipase. *Eur J Biochem* 1994; **219**: 905-914 [PMID: 8112342 DOI: 10.1111/j.1432-1033.1994.tb18572.x]
22 **Du H**, Sheriff S, Bezerra J, Leonova T, Grabowski GA. Molecular and enzymatic analyses of lysosomal acid lipase in cholesteryl ester storage disease. *Mol Genet Metab* 1998; **64**: 126-134 [PMID: 9705237 DOI: 10.1006/mgme.1998.2707]
23 **Sheriff S**, Du H, Grabowski GA. Characterization of lysosomal acid lipase by site-directed mutagenesis and heterologous expression. *J Biol Chem* 1995; **270**: 27766-27772 [PMID: 7499245 DOI: 10.1074/jbc.270.46.27766]
24 **Du H**, Cameron TL, Garger SJ, Pogue GP, Hamm LA, White E, Hanley KM, Grabowski GA. Wolman disease/cholesteryl ester storage disease: efficacy of plant-produced human lysosomal acid lipase in mice. *J Lipid Res* 2008; **49**: 1646-1657 [PMID: 18413899 DOI: 10.1194/jlr.M700482-JLR200]
25 **Zschenker O**, Bähr C, Hess UF, Ameis D. Systematic mutagenesis of potential glycosylation sites of lysosomal acid lipase. *J Biochem* 2005; **137**: 387-394 [PMID: 15809341 DOI: 10.1093/jb/mvi043]
26 **Komaromy MC**, Schotz MC. Cloning of rat hepatic lipase cDNA: evidence for a lipase gene family. *Proc Natl Acad Sci USA* 1987; **84**: 1526-1530 [PMID: 3470738 DOI: 10.1073/pnas.84.6.1526]
27 **Lohse P**, Lohse P, Chahrokh-Zadeh S, Seidel D. Human lysosomal acid lipase/cholesteryl ester hydrolase and human gastric lipase: site-directed mutagenesis of Cys227 and Cys236 results in substrate-dependent reduction of enzymatic activity. *J Lipid Res* 1997; **38**: 1896-1905 [PMID: 9323599]
28 **Roussel A**, Canaan S, Egloff MP, Rivière M, Dupuis L, Verger R, Cambillau C. Crystal structure of human gastric lipase and model of lysosomal acid lipase, two lipolytic enzymes of medical interest. *J Biol Chem* 1999; **274**: 16995-17002 [PMID: 10358049 DOI: 10.1074/jbc.274.24.16995]
29 **Du H**, Duanmu M, Witte D, Grabowski GA. Targeted disruption of the mouse lysosomal acid lipase gene: long-term survival with massive cholesteryl ester and triglyceride storage. *Hum Mol Genet* 1998; **7**: 1347-1354 [PMID: 9700186 DOI: 10.1093/hmg/7.9.1347]
30 **Yan C**, Lian X, Dai Y, Wang X, Qu P, White A, Qin Y, Du H. Gene delivery by the hSP-B promoter to lung alveolar type II epithelial cells in LAL-knockout mice through bone marrow mesenchymal stem cells. *Gene Ther* 2007; **14**: 1461-1470 [PMID: 17700706 DOI: 10.1038/sj.gt.3303006]
31 **Lian X**, Yan C, Qin Y, Knox L, Li T, Du H. Neutral lipids and peroxisome proliferator-activated receptor-{gamma} control pulmonary gene expression and inflammation-triggered pathogenesis in lysosomal acid lipase knockout mice. *Am J Pathol* 2005; **167**: 813-821 [PMID: 16127159 DOI: 10.1016/S0002-9440(10)62053-6]
32 **Yan C**, Lian X, Li Y, Dai Y, White A, Qin Y, Li H, Hume DA, Du H. Macrophage-specific expression of human lysosomal acid lipase corrects inflammation and pathogenic phenotypes in lal-/- mice. *Am J Pathol* 2006; **169**: 916-926 [PMID:

16936266 DOI: 10.2353/ajpath.2006.051327]
33 **Qu P**, Du H, Wilkes DS, Yan C. Critical roles of lysosomal acid lipase in T cell development and function. *Am J Pathol* 2009; **174**: 944-956 [PMID: 19179613 DOI: 10.2353/ajpath.2009.080562]
34 **Qu P**, Yan C, Blum JS, Kapur R, Du H. Myeloid-specific expression of human lysosomal acid lipase corrects malformation and malfunction of myeloid-derived suppressor cells in lal-/- mice. *J Immunol* 2011; **187**: 3854-3866 [PMID: 21900179 DOI: 10.4049/jimmunol.1003358]
35 **Du H**, Levine M, Ganesa C, Witte DP, Cole ES, Grabowski GA. The role of mannosylated enzyme and the mannose receptor in enzyme replacement therapy. *Am J Hum Genet* 2005; **77**: 1061-1074 [PMID: 16380916]
36 **Du H**, Schiavi S, Levine M, Mishra J, Heur M, Grabowski GA. Enzyme therapy for lysosomal acid lipase deficiency in the mouse. *Hum Mol Genet* 2001; **10**: 1639-1648 [PMID: 11487567]
37 **Balwani M**, Breen C, Enns GM, Deegan PB, Honzík T, Jones S, Kane JP, Malinova V, Sharma R, Stock EO, Valayannopoulos V, Wraith JE, Burg J, Eckert S, Schneider E, Quinn AG. Clinical effect and safety profile of recombinant human lysosomal acid lipase in patients with cholesteryl ester storage disease. *Hepatology* 2013; **58**: 950-957 [PMID: 23348766 DOI: 10.1002/hep.26289]
38 **Du H**, Heur M, Duanmu M, Grabowski GA, Hui DY, Witte DP, Mishra J. Lysosomal acid lipase-deficient mice: depletion of white and brown fat, severe hepatosplenomegaly, and shortened life span. *J Lipid Res* 2001; **42**: 489-500 [PMID: 11290820]
39 **Lian X**, Yan C, Yang L, Xu Y, Du H. Lysosomal acid lipase deficiency causes respiratory inflammation and destruction in the lung. *Am J Physiol Lung Cell Mol Physiol* 2004; **286**: L801-L807 [PMID: 14644759]
40 **Ding X**, Du H, Yoder MC, Yan C. Critical role of the mTOR pathway in development and function of myeloid-derived suppressor cells in lal-/- mice. *Am J Pathol* 2014; **184**: 397-408 [PMID: 24287405 DOI: 10.1016/j.ajpath.2013.10.015]
41 **Qu P**, Shelley WC, Yoder MC, Wu L, Du H, Yan C. Critical roles of lysosomal acid lipase in myelopoiesis. *Am J Pathol* 2010; **176**: 2394-2404 [PMID: 20348241 DOI: 10.2353/ajpath.2010.091063]
42 **Talmadge JE**, Gabrilovich DI. History of myeloid-derived suppressor cells. *Nat Rev Cancer* 2013; **13**: 739-752 [PMID: 24060865 DOI: 10.1038/nrc3581]
43 **Okwan-Duodu D**, Umpierrez GE, Brawley OW, Diaz R. Obesity-driven inflammation and cancer risk: role of myeloid derived suppressor cells and alternately activated macrophages. *Am J Cancer Res* 2013; **3**: 21-33 [PMID: 23359288]
44 **Cuenca AG**, Delano MJ, Kelly-Scumpia KM, Moreno C, Scumpia PO, Laface DM, Heyworth PG, Efron PA, Moldawer LL. A paradoxical role for myeloid-derived suppressor cells in sepsis and trauma. *Mol Med* 2011; **17**: 281-292 [PMID: 21085745 DOI: 10.2119/molmed.2010.00178]
45 **Goh C**, Narayanan S, Hahn YS. Myeloid-derived suppressor cells: the dark knight or the joker in viral infections? *Immunol Rev* 2013; **255**: 210-221 [PMID: 23947357 DOI: 10.1111/imr.12084]
46 **Natarajan S**, Thomson AW. Tolerogenic dendritic cells and myeloid-derived suppressor cells: potential for regulation and therapy of liver auto- and alloimmunity. *Immunobiology* 2010; **215**: 698-703 [PMID: 20605054 DOI: 10.1016/j.imbio.2010.05.024]
47 **Dilek N**, Vuillefroy de Silly R, Blancho G, Vanhove B. Myeloid-derived suppressor cells: mechanisms of action and recent advances in their role in transplant tolerance. *Front Immunol* 2012; **3**: 208 [PMID: 22822406 DOI: 10.3389/fimmu.2012.00208]
48 **Yin B**, Ma G, Yen CY, Zhou Z, Wang GX, Divino CM, Casares S, Chen SH, Yang WC, Pan PY. Myeloid-derived suppressor cells prevent type 1 diabetes in murine models. *J Immunol* 2010; **185**: 5828-5834 [PMID: 20956337 DOI: 10.4049/jimmunol.0903636]
49 **Morales JK**, Saleem SJ, Martin RK, Saunders BL, Barnstein BO, Faber TW, Pullen NA, Kolawole EM, Brooks KB, Norton SK, Sturgill J, Graham L, Bear HD, Urban JF, Lantz CS, Conrad DH, Ryan JJ. Myeloid-derived suppressor cells enhance IgE-mediated mast cell responses. *J Leukoc Biol* 2014; **95**: 643-650 [PMID: 24338630 DOI: 10.1189/jlb.0913510]
50 **Ostanin DV**, Bhattacharya D. Myeloid-derived suppressor cells in the inflammatory bowel diseases. *Inflamm Bowel Dis* 2013; **19**: 2468-2477 [PMID: 23811636 DOI: 10.1097/MIB.0b013e3182902b11]
51 **Gabrilovich DI**, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009; **9**: 162-174 [PMID: 19197294 DOI: 10.1038/nri2506]
52 **Ostrand-Rosenberg S**, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 2009; **182**: 4499-4506 [PMID: 19342621 DOI: 10.4049/jimmunol.0802740]
53 **Sica A**, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. *J Clin Invest* 2007; **117**: 1155-1166 [PMID: 17476345]
54 **Lindau D**, Gielen P, Kroesen M, Wesseling P, Adema GJ. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. *Immunology* 2013; **138**: 105-115 [PMID: 23216602 DOI: 10.1111/imm.12036]
55 **Sasmono RT**, Oceandy D, Pollard JW, Tong W, Pavli P, Wainwright BJ, Ostrowski MC, Himes SR, Hume DA. A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* 2003; **101**: 1155-1163 [PMID: 12393599 DOI: 10.1182/blood-2002-02-0569]
56 **Weissman IL**, Shizuru JA. The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood* 2008; **112**: 3543-3553 [PMID: 18948588 DOI: 10.1182/blood-2008-08-078220]
57 **Blank U**, Karlsson G, Karlsson S. Signaling pathways governing stem-cell fate. *Blood* 2008; **111**: 492-503 [PMID: 17914027 DOI: 10.1182/blood-2007-07-075168]
58 **Li Y**, Du H, Qin Y, Roberts J, Cummings OW, Yan C. Activation of the signal transducers and activators of the transcription 3 pathway in alveolar epithelial cells induces inflammation and adenocarcinomas in mouse lung. *Cancer Res* 2007; **67**: 8494-8503 [PMID: 17875688 DOI: 10.1158/0008-5472.CAN-07-0647]
59 **Wu L**, Du H, Li Y, Qu P, Yan C. Signal transducer and activator of transcription 3 (Stat3C) promotes myeloid-derived suppressor cell expansion and immune suppression during lung tumorigenesis. *Am J Pathol* 2011; **179**: 2131-2141 [PMID: 21864492 DOI: 10.1016/j.ajpath.2011.06.028]
60 **Wang HY**, Wang RF. Regulatory T cells and cancer. *Curr Opin Immunol* 2007; **19**: 217-223 [PMID: 17306521 DOI: 10.1016/j.coi.2007.02.004]
61 **Neves SR**, Ram PT, Iyengar R. G protein pathways. *Science* 2002; **296**: 1636-1639 [PMID: 12040175 DOI: 10.1126/science.1071550]
62 **Gavi S**, Shumay E, Wang HY, Malbon CC. G-protein-coupled receptors and tyrosine kinases: crossroads in cell signaling and regulation. *Trends Endocrinol Metab* 2006; **17**: 48-54 [PMID: 16460957 DOI: 10.1016/j.tem.2006.01.006]
63 **Konstantinopoulos PA**, Karamouzis MV, Papavassiliou AG. Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. *Nat Rev Drug Discov* 2007; **6**: 541-555 [PMID: 17585331 DOI: 10.1038/nrd2221]
64 **Yan C**, Ding X, Dasgupta N, Wu L, Du H. Gene profile of myeloid-derived suppressive cells from the bone marrow of lysosomal acid lipase knock-out mice. *PLoS One* 2012; **7**: e30701 [PMID: 22383970 DOI: 10.1371/journal.pone.0030701]
65 **Malumbres M**, Barbacid M. Cell cycle, CDKs and cancer: a

changing paradigm. *Nat Rev Cancer* 2009; **9**: 153-166 [PMID: 19238148 DOI: 10.1038/nrc2602]
66 **Ovaa H**. Active-site directed probes to report enzymatic action in the ubiquitin proteasome system. *Nat Rev Cancer* 2007; **7**: 613-620 [PMID: 17646866 DOI: 10.1038/nrc2128]
67 **Guertin DA**, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell* 2007; **12**: 9-22 [PMID: 17613433]
68 **Bousquet M**, Recher C, Queleen C, Demur C, Payrastre B, Brousset P. Assessment of somatic mutations in phosphatidylinositol 3-kinase gene in human lymphoma and acute leukaemia. *Br J Haematol* 2005; **131**: 411-413 [PMID: 16225664]
69 **Zoncu R**, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2011; **12**: 21-35 [PMID: 21157483 DOI: 10.1038/nrm3025]
70 **Korolchuk VI**, Saiki S, Lichtenberg M, Siddiqi FH, Roberts EA, Imarisio S, Jahreiss L, Sarkar S, Futter M, Menzies FM, O' Kane CJ, Deretic V, Rubinsztein DC. Lysosomal positioning coordinates cellular nutrient responses. *Nat Cell Biol* 2011; **13**: 453-460 [PMID: 21394080 DOI: 10.1038/ncb2204]
71 **Thomson AW**, Turnquist HR, Raimondi G. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* 2009; **9**: 324-337 [PMID: 19390566 DOI: 10.1038/nri2546]
72 **Weichhart T**, Säemann MD. The multiple facets of mTOR in immunity. *Trends Immunol* 2009; **30**: 218-226 [PMID: 19362054 DOI: 10.1016/j.it.2009.02.002]
73 **Heitman J**, Movva NR, Hall MN. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* 1991; **253**: 905-909 [PMID: 1715094]
74 **Sabatini DM**, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* 1994; **78**: 35-43 [PMID: 7518356]
75 **Brown EJ**, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, Schreiber SL. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* 1994; **369**: 756-758 [PMID: 8008069]
76 **Yan C**, Du H. Alveolus formation: what have we learned from genetic studies? *J Appl Physiol* 2004; **97**: 1543-1548 [PMID: 15358757]
77 **Kiss M**, Czimmerer Z, Nagy L. The role of lipid-activated nuclear receptors in shaping macrophage and dendritic cell function: From physiology to pathology. *J Allergy Clin Immunol* 2013; **132**: 264-286 [PMID: 23905916 DOI: 10.1016/j.jaci.2013.05.044]
78 **Nagy L**, Szanto A, Szatmari I, Széles L. Nuclear hormone receptors enable macrophages and dendritic cells to sense their lipid environment and shape their immune response. *Physiol Rev* 2012; **92**: 739-789 [PMID: 22535896 DOI: 10.1152/physrev.00004.2011]
79 **Jiang C**, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* 1998; **391**: 82-86 [PMID: 9422509]
80 **Ricote M**, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 1998; **391**: 79-82 [PMID: 9422508]
81 **Wu L**, Yan C, Czader M, Foreman O, Blum JS, Kapur R, Du H. Inhibition of PPARγ in myeloid-lineage cells induces systemic inflammation, immunosuppression, and tumorigenesis. *Blood* 2012; **119**: 115-126 [PMID: 22053106 DOI: 10.1182/blood-2011-06-363093]
82 **Page-McCaw A**, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* 2007; **8**: 221-233 [PMID: 17318226]
83 **Kessenbrock K**, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 2010; **141**: 52-67 [PMID: 20371345 DOI: 10.1016/j.cell.2010.03.015]
84 **Marco M**, Fortin C, Fulop T. Membrane-type matrix metalloproteinases: key mediators of leukocyte function. *J Leukoc Biol* 2013; **94**: 237-246 [PMID: 23695309 DOI: 10.1189/jlb.0612267]
85 **Werb Z**, Gordon S. Elastase secretion by stimulated macrophages. Characterization and regulation. *J Exp Med* 1975; **142**: 361-377 [PMID: 167096]
86 **Gronski TJ**, Martin RL, Kobayashi DK, Walsh BC, Holman MC, Huber M, Van Wart HE, Shapiro SD. Hydrolysis of a broad spectrum of extracellular matrix proteins by human macrophage elastase. *J Biol Chem* 1997; **272**: 12189-12194 [PMID: 9115292]
87 **Shapiro SD**, Kobayashi DK, Ley TJ. Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages. *J Biol Chem* 1993; **268**: 23824-23829 [PMID: 8226919]
88 **Hautamaki RD**, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 1997; **277**: 2002-2004 [PMID: 9302297]
89 **Hofmann HS**, Hansen G, Richter G, Taege C, Simm A, Silber RE, Burdach S. Matrix metalloproteinase-12 expression correlates with local recurrence and metastatic disease in non-small cell lung cancer patients. *Clin Cancer Res* 2005; **11**: 1086-1092 [PMID: 15709175]
90 **Qu P**, Du H, Wang X, Yan C. Matrix metalloproteinase 12 overexpression in lung epithelial cells plays a key role in emphysema to lung bronchioalveolar adenocarcinoma transition. *Cancer Res* 2009; **69**: 7252-7261 [PMID: 19706765 DOI: 10.1158/0008-5472.CAN-09-0577]
91 **MacIvor DM**, Shapiro SD, Pham CT, Belaaouaj A, Abraham SN, Ley TJ. Normal neutrophil function in cathepsin G-deficient mice. *Blood* 1999; **94**: 4282-4293 [PMID: 10590073]
92 **Shapiro SD**, Senior RM. Matrix metalloproteinases. Matrix degradation and more. *Am J Respir Cell Mol Biol* 1999; **20**: 1100-1102 [PMID: 10340927]
93 **Lee G**, Walser TC, Dubinett SM. Chronic inflammation, chronic obstructive pulmonary disease, and lung cancer. *Curr Opin Pulm Med* 2009; **15**: 303-307 [PMID: 19417670 DOI: 10.1097/MCP.0b013e32832c975a]
94 **Sohal SS**, Ward C, Danial W, Wood-Baker R, Walters EH. Recent advances in understanding inflammation and remodeling in the airways in chronic obstructive pulmonary disease. *Expert Rev Respir Med* 2013; **7**: 275-288 [PMID: 23734649 DOI: 10.1586/ers.13.26]
95 **Gebe JA**, Llewellyn M, Hoggatt H, Aruffo A. Molecular cloning, genomic organization and cell-binding characteristics of mouse Spalpha. *Immunology* 2000; **99**: 78-86 [PMID: 10651944]
96 **Miyazaki T**, Hirokami Y, Matsuhashi N, Takatsuka H, Naito M. Increased susceptibility of thymocytes to apoptosis in mice lacking AIM, a novel murine macrophage-derived soluble factor belonging to the scavenger receptor cysteine-rich domain superfamily. *J Exp Med* 1999; **189**: 413-422 [PMID: 9892623]
97 **Qu P**, Du H, Li Y, Yan C. Myeloid-specific expression of Api6/AIM/Sp alpha induces systemic inflammation and adenocarcinoma in the lung. *J Immunol* 2009; **182**: 1648-1659 [PMID: 19155514]
98 **Kao J**, Ko EC, Eisenstein S, Sikora AG, Fu S, Chen SH. Targeting immune suppressing myeloid-derived suppressor cells in oncology. *Crit Rev Oncol Hematol* 2011; **77**: 12-19 [PMID: 20304669 DOI: 10.1016/j.critrevonc.2010.02.004]

P- Reviewer: Gopinath SCB, Schuurman HJ, Saeki K
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Wang CH

World Journal of Immunology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5411/wji.v4.i2.52

World J Immunol 2014 July 27; 4(2): 52-62
ISSN 2219-2824 (online)

REVIEW

Gut immune response in the presence of hepatitis C virus infection

Helal F Hetta, Minesh J Mehta, Mohamed Tarek M Shata

Helal F Hetta, Minesh J Mehta, Mohamed Tarek M Shata, Department of Internal Medicine, Division of Digestive Diseases, University of Cincinnati, Cincinnati, OH 45267, United States
Helal F Hetta, Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut 71515, Egypt
Author contributions: All the authors contributed to this paper.
Supported by Egyptian Government Scholarship for Helal Hetta; and Merck Investigator Initiated Studies (IIS) IISP, No. 40458 (Shata)
Correspondence to: Mohamed Tarek M Shata, MD, PhD, Department of Internal Medicine, Division of Digestive Diseases, University of Cincinnati, 231 Albert B. Sabin Way, Cincinnati, OH 45267, United States. mohamed.shata@uc.edu
Telephone: +1-513-5586110 Fax: +1-513-5581744
Received: April 2, 2014 Revised: May 22, 2014
Accepted: June 20, 2014
Published online: July 27, 2014

Abstract

Hepatitis C virus (HCV) is an important etiologic agent of hepatitis and a major cause of chronic liver infection that often leads to cirrhosis, fibrosis and hepatocellular carcinoma. Although, HCV is a hepatotropic virus, there is strong evidence that HCV could replicate extrahepatic in the gastrointestinal tissue which could serve as a reservoir for HCV. The outcome of HCV infection depends mainly on the host innate and adaptive immune responses. Innate immunity against HCV includes mainly nuclear factor cells and activation of IFN-related genes. There is an immunologic link between the gut and the liver through a population of T-cells that are capable of homing to both the liver and gut *via* the portal circulation. However, little is known on the role of Gut immune response in HCV. In this review we discussed the immune regulation of Gut immune cells and its association with HCV pathogenesis, various outcomes of anti-HCV therapy, viral persistence and degree of liver inflammation. Additionally, we investigated the relationship between Gut immune responses to HCV and IL28B genotypes, which were identified as a strong predictor for HCV pathogenesis and treatment outcome after acute infection.

Key words: Hepatitis C virus; Colonic T_{reg}; Mucosal; Immune regulation; Liver inflammation; Interleukin-28B

Core tip: Chronic hepatitis C (CHC) is a global worldwide health problem with approximately 200 million people worldwide infected with hepatitis C virus (HCV). It is also a major cause of chronic liver infection that often leads to chronic hepatitis which may progress to cirrhosis, fibrosis and finally hepatocellular carcinoma. In CHC, immune responses play an important role in HCV pathogenesis and responses to therapy. Intrahepatic immune responses to HCV are highly regulated. There is a clear relationship between hepatic immune responses and mucosal immune response in the gut. Additionally, genetic immunological markers have been proposed to predict response to HCV treatment, and outcome of infection.

Hetta HF, Mehta MJ, Shata MTM. Gut immune response in the presence of hepatitis C virus infection. *World J Immunol* 2014; 4(2): 52-62 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i2/52.htm DOI: http://dx.doi.org/10.5411/wji.v4.i2.52

INTRODUCTION

Hepatitis C virus (HCV) was first identified by Harvey Alter in 1978 and named non-A, non-B hepatitis[1], and cloned by Houghton in 1986[2]. HCV is a single-stranded, positive sense RNA virus belonging to *Hepacivirus* group in the family Flaviviridae[3]. There are 6 HCV genotypes. Due to the low fidelity and lack of proofreading of HCV

polymerase enzymes used for viral genome amplification, multiple mutations occur within a genotype to produce quasi-species[4,5].

Chronic hepatitis C infection (CHC) is a global worldwide health care problem with an increasing burden year-by-year[6-8]. The World Health Organization estimates that approximately 200 million people worldwide are infected with HCV[9]. It is also a major cause of chronic liver infection that often leads to chronic hepatitis which may progress to cirrhosis, fibrosis and finally hepatocellular carcinoma[3,10].

HCV is one of the most important etiologic agents of post transfusion hepatitis. HCV is usually spread by sharing infected needles with a carrier, from receiving infected blood, and from accidental exposure to infected blood. Some people acquire the infection through non parenteral means that have not been fully defined, but include sexual transmission in persons with high risk behaviors[11]. It is not reported that HCV can spread orally by food, water, breast feeding, or by normal social contact as sneezing, coughing, hugging, sharing eating utensils or drinking glasses[12]. Mother-to-baby transmission is rare and needs a high viremia as found in HIV co-infection[13].

HCV VIROLOGY

HCV is a single stranded RNA virus which produces negative strand RNA as a replicative intermediate. The HCV genome is about 9.6 kb in length. During HCV replication cycle, one large precursor protein is synthesized from an open reading frame then cleaved to produce 10 proteins including three structural proteins which are Core, two envelope proteins (E1 and E2)[3], and P7 which results from cleavage of E2 protein[14]. The other six proteins that are not in the viral particle called non-structural proteins (NS) including NS2, NS3, NS4A, NS4B, NS5A, and NS5B[3]. Non-structural (NS) proteins are not found in the virion, therefore, presence of NS proteins inside cells suggests that HCV replication occurred in those cells[3]. Replication of HCV involves converting the viral genomic positive strand into a negative strand, and then back to the genomic strand. Thus, the presence of the negative strand strongly suggests that replication[15].

HCV REPLICATION

HCV is primarily a hepatotropic virus[15]. However, a broad spectrum of extra-hepatic manifestations may be associated with HCV infection, including mixed cryoglobulinemia, non-Hodgkin's lymphoma, arthralgia, paresthesia, myalgia, pruritis, cutaneous vasculitis, glomerulonephritis, neuropathy and lymphoproliferative disorders[16,17].

HCV was believed to infect only hepatocytes[3]. However, recent studies have reported HCV infection of other cell types[15,18-21]. In fact, viral replication has been reported in B cells, T cells, monocytes, macrophages, and macrophage-like cells such as Kupffer cells, dendritic cells (DCs), renal cells, thyroid cells, and gastric cells. There is mounting evidence that these cells could represent replicative compartments for the virus[3,22,23]. In addition, it has been proposed that peripheral blood monocytes (PBMC) could be the source of recurrent HCV infection after liver transplantation[24]. Despite these reports, extra-hepatic replication of HCV is still controversial by some investigators. However, the importance of extra-hepatic HCV replication in HCV pathogenesis is clear. Extra-hepatic compartments might serve as reservoirs for HCV, and hence HCV persistence, reactivation after antiviral therapy and also may contribute to the HCV extra-hepatic manifestations[24].

HCV IN THE GUT

There is a molecular evidence that HCV may infect and replicate in oral mucosa and gastric cells[23]. Moreover, HCV seems to be involved in development of B-cell non-Hodgkin's lymphoma of the gastric mucosa[25]. Miglioresi *et al*[26], reported that Gut mucosa may serve as possible reservoir for HCV relapse after viral clearance. They analyzed HCV gastric localization in 15 patients and compared their levels of viremia with the status of HCV in gastric biopsy specimens and PBMCs. In that study, all 15 patients with positive viremia were positive for HCV RNA on Gut tissue and PBMCs. In 2 patients, HCV RNA was positive on serum, negative at Gut biopsy but their PBMCs were positive. Two patients with negative viremia and PBMCs after antiviral treatment were positive for HCV RNA on gastric sample and eventually relapsed (after 6 and 18 wk). The finding of a positive hidden compartment for HCV and simultaneous negative viremia had previously reported in HCV infected liver without detectable viremia[27]. Replication of HCV in gastrointestinal tissue represents a continuous new source as an extra-hepatic reservoir of viral particles for re-infection of hepatocytes[26].

IMMUNE RESPONSE TO HCV

Systemic immune responses

The immune response against HCV involves innate and adaptive immunity[9]. Innate immunity against HCV is mediated by several innate immune effector cells such as NK cells, and activation of the interferons-stimulated genes (ISGs) response[28]. Recent studies have revealed that the *IL28B* gene locus, which codes for a type Ⅲ interferon is a critical locus for outcome after acute infection[29], and response to therapy[29,30]. However, HCV may develop several strategies to overcome these responses. For example, viral NS3 and NS4a protease can cause disruption of important components of type I interferon activation cascade through inactivation of several ISGs[31,32].

Adaptive immunity against HCV is mediated by both humoral and cellular immune responses. Most HCV-

infected individuals develop antibodies against HCV, regardless of the outcome of infection. Few of these antibodies can neutralize viral particles and may limit viral spread[33]. However, neutralizing antibodies have a limited role in most of the infected patients due to the high replication and mutation rate of HCV[34]. In fact, HCV clearance had been observed in some patients in the absence of neutralising antibodies[35]. Therefore, despite the potential protective role of innate and humoral immunity in the outcome of infection, it is clear that protection and viral clearance depend primarily on cellular adaptive immune responses through a complex interplay between $CD4^+$ and $CD8^+$ T-cell responses[9]. Unfortunately, in some patients, cellular immune responses are inadequate and fail to clear the infection with a subsequent viral persistence[9]. Fully functional virus-specific $CD4^+$T-cell responses are detectable in patients who cleared infection[9,36-38]. The role of HCV-specific $CD4^+$T-cell was further supported by the finding of *in vivo* depletion of $CD4^+$T cells from HCV-recovered chimpanzees was associated with viral persistence[38]. Moreover, several studies have shown that HCV-specific $CD8^+$T-cells derived from the peripheral blood or liver are functionally impaired and display a reduced ability to proliferate or secrete anti-viral cytokines such as IFN-γ [39-41]. The mechanisms contributing to $CD8^+$T cell exhaustion in HCV are not fully understood, however, it may be partially explained by the intrinsic regulatory pathways such as signals mediated by the inhibitory receptor PD-1[40,42-45] and extrinsic regulatory pathways as regulatory T cells (T_{reg}) or secretion of immunoregulatory cytokines such as IL-10[46-51]. Ultimately, the outcome of HCV infection, viral persistence or clearance, is determined by the host immune response[9,52,53]. Additionally, sustained HCV-specific cytotoxic T cell responses in the liver have been associated with the development of hepatic immunopathology and liver necrosis which may lead to liver cirrhosis[52,53]. The mechanisms that mediate liver inflammation and damage in CHC are not yet fully elucidated[9,54]. One of the potential mechanisms that might modulate HCV-specific immune responses is T_{reg} cells which are a subtype of T cells that play a fundamental role in maintaining immune homeostasis and the balance between the tissue-damaging and protective effects of the immune response[54-56]. It is characterized by the expression of a unique transcription factor Forkhead box protein P3 (FoxP3), which is highly expressed in the nucleus of T_{reg} cells and is generally accepted as the single best marker to quantify T_{reg} cells[53,56-58]. In cases with CHC, it was reported that the frequency of T_{reg} cells were negatively correlated with the degree of necro-inflammatory scores and their frequency is higher than that in healthy individuals[47,59,60]. Thus, T_{reg} cells appear to assist in the maintenance of chronicity by inhibition of anti-HCV immune responses and consequently attenuate the intrahepatic tissue-damaging response to infection[49,53].

MUCOSAL (GUT) IMMUNE RESPONSE IN HCV

The mucosal immune system is considered the first line of defense that reduces the need for elimination of exogenous invading antigens by pro-inflammatory immune response[61]. The mucosal immune system maintains homeostasis through evolution of two layers of adaptive non-inflammatory defense; the first strategy is immune exclusion by secretory IgA (and IgM) antibodies to limit epithelial contact and penetration of invading microorganisms and other potentially dangerous antigens[61], and the second strategy is oral tolerance by development of immunosuppressive mechanisms to inhibit over-reaction against food antigens and commensal bacteria[62]. Oral tolerance depends mainly on the induction of T_{reg} cells in mesenteric lymph nodes to which mucosal DCs carry and present food and commensal microbial antigens[63]. Gut induced tolerance include other suppressive mechanisms to ensure that persistent food allergy is relatively rare[64].

Some pathogens and food antigens could enter the liver via the portal circulation[65] within 2 h of ingestion[66] and presented on liver endothelial cells. The liver is critical in the regulation of immune responses to pathogens entering *via* portal circulation[67]. It receives 75% of its blood supply from the portal vein, which drains the gut. Oral tolerance is usually lost in case of a portal-systemic shunt, which allows portal blood to bypass the liver and goes directly from the gut to the systemic circulation[67,68].

To understand the interactions between the immune responses in the Gut and the liver during HCV infection, we have to dissect the immune responses in each organ. The intestinal immune system can be divided into inductive and effector sites based upon their anatomical and functional properties[61,63]. Inductive sites include the gut-associated lymphoid tissues (GALT) such as Peyer's patches (PP) and isolated lymphoid follicles and the mesenteric lymph nodes (mLNs). The GALT contains a wide variety of cells, such as Microfold (M) cells, DCs, intraepithelial lymphocytes (IEL), macrophages and T_{reg} cells[61]. The main effector sites of the intestinal immune system are the lamina propria (LP) and epithelium, which harbor large populations of activated T cells and antibody-secreting plasma cells. The LP may also contribute to the induction of tolerance. It is a site of antigen uptake and loading of the migratory DCs that encounter naïve T cells in the mLNs[61]. Antigen are up-taken by absorptive epithelial and M cells in the mucosal inductive sites or directly captured by professional APCs (including DCs, Macrophage and B lymphocytes)[69]. M cells take up molecules and particles from the gut lumen by endocytosis or phagocytosis then sample them to the immune cells. Antigens are transported through M cells by the process of transcytosis. The cell membrane at the base of M cells is folded around lymphocytes and dendritic cells within the Peyer's patches[69]. M cells present the antigen to conventional $CD4^+$ and $CD8^+$ αβ T cells at the inductive site.

At the same time, epithelial cells may process and present certain antigens directly to neighboring intraepithelial T cells such as NKT cells and γδ T cells which are T cells with limited repertoire diversity[69]. Naive B and T cells enter GALT and are primed to become memory/effector B and T cells, then migrate from GALT to mesenteric blood and the liver or to the lymph nodes *via* lymph and then *via* thoracic duct to peripheral blood for subsequent extravasation at mucosal effector sites. A system of Gut-specific lymphocyte trafficking has been evolved to target lymphocyte to the area of injury or infection through vascular adhesion molecules and chemokines. Thus, the endothelial cells act as a local gatekeeper for mucosal immunity[61]. Under normal physiological conditions enteric antigens are presented to naïve lymphocytes in the draining mesenteric lymph nodes. Lymphocytes activated by gut dendritic cells express a gut-homing phenotype characterized by expression of the chemokine receptor CCR9 and the integrin α4β7 which direct the migration of the activated lymphocytes back to gut tissue where their respective ligands CCL25 and MAdCAM-1 are expressed[67,70]. Lymphocytes that are primed to hepatic antigens acquire expression of adhesion molecules that direct them to traffic to the liver by interacting with molecules expressed on hepatic endothelium such as VAP-1.

EFFECTOR MECHANISMS OF THE GUT IMMUNE RESPONSES

Innate immune system in the gut includes the lining epithelium which provides barrier function, mechanical cleaning and defensins which act as chemical antimicrobial factors[71]. The gut mucosa contains a number of other cells as part of the innate immune system, including phagocytic neutrophils and macrophages, DCs, NK cells and mast cells. These cells contribute significantly to host defense against pathogens[22] and also initiate adaptive mucosal immune responses[69,72].

The adaptive humoral immune defense at the gut mucosal surfaces is mainly mediated by secretory IgA (sIgA) antibody, which is the ideal antibody for functioning in mucosal secretions due to its resistance to proteases[61]. sIgA plays a protective role against a variety of foreign antigens such as food antigens, toxins, bacteria and viruses[72]. slgA blocks the access of potentially allergenic molecules derived from food or drugs[73]. Because some dietary antigen is clearly absorbed by normal subjects, the importance of sIgA antibody may lie in reducing the amount of antigen that gains access to the lamina propria[73,74]. sIgA can neutralize biologically active antigens as bacteria, toxins, enzymes and viruses. The effectiveness of sIgA as a neutralizing antibody against viruses is shown for example in the responses to oral live-attenuated poliovirus vaccine where protection correlates with levels of secretory antibody[75]. Additionally, sIgA is an efficient agglutinin that can prevent adherence of pathogenic bacteria to the epithelial surfaces and enhance the antibacterial efficiency of other effector immune system; sIgA has bactericidal potential by cooperation with complement and lysozyme and also can act as opsonin. However, the role of sIgA during HCV infection is limited.

The development of IgA immune response against mucosal pathogens and soluble protein antigens is dependent on T helper cells[76]. Mucosal T cells produce large amounts of transforming growth factor (TGF)-β, interleukin (IL)-10 and IL-4 to promote B-cell isotype class switching to IgA[77,78]. Additionally, muco-epithelial cells, and T_{reg} cells are the major sources of TGF-β and IL-10, suggesting that cooperation between neighboring lymphocytes and epithelial cells in the mucosal microenvironment is pivotal to promote B-cell switch to IgA and differentiation into IgA-committed B cells[69].

One of the important cellular immune defense at the gut mucosal surfaces is mainly mediated by cytotoxic T lymphocyte (CTL) responses[69]. It is reported that mucosal CTLs are crucial for the immune clearance of pathogens in several animal models of infection with enteric viruses like Rota virus[79] and intracellular parasites[80]. Besides CTLs, induced IFN-γ producing $CD4^+$ T cells, have been found to be important for mucosal immune defense to both viral and bacterial infections[69].

REGULATORY MECHANISMS OF GUT IMMUNE RESPONSE AND ORAL TOLERANCE

Gut immune response is controlled by the local microenvironment, the nature of the antigen and the type of APCs. In case of foreign food proteins and non-pathogen antigens, the default pathway for mucosal DCs and other APCs is to generate T_H2 and various regulatory T cell types of responses mainly T_{reg}[81], and Th_{17} cells[82] which usually leads to down-regulatory or active suppression of systemic immunity (oral tolerance). On the other hand, antigens, most pathogens harboring motifs which could bind to Toll-like receptor (TLR), and be sensed by mucosal APCs as 'danger signals' and pro-inflammatory conditions in general favor the development of stronger and broader immune responses but do not lead to oral tolerance[81,83,84]. Oral tolerance can be achieved through different mechanisms, including anergy, activation-induced cell death and most important, the induction of regulatory T cells[69,85]. Anergy of antigen-specific T cells has been reported after ingestion of large quantities of soluble proteins[86], and deletion of specific T cells only after mucosal administration of massive, non-physiological antigen doses[87]. Induction of regulatory T cells after mucosal delivery of antigens has been reported and received major attention given the potential of manipulating these regulatory cells as therapeutic agents in immune-mediated diseases[69].

Regulatory T cells includes: (1) $CD4^+CD45RB^{low}$ Tr1 cells that function through the production of IL-10 to suppresses antigen-specific T cell responses and actively down-regulates a pathological immune response[88]; (2)

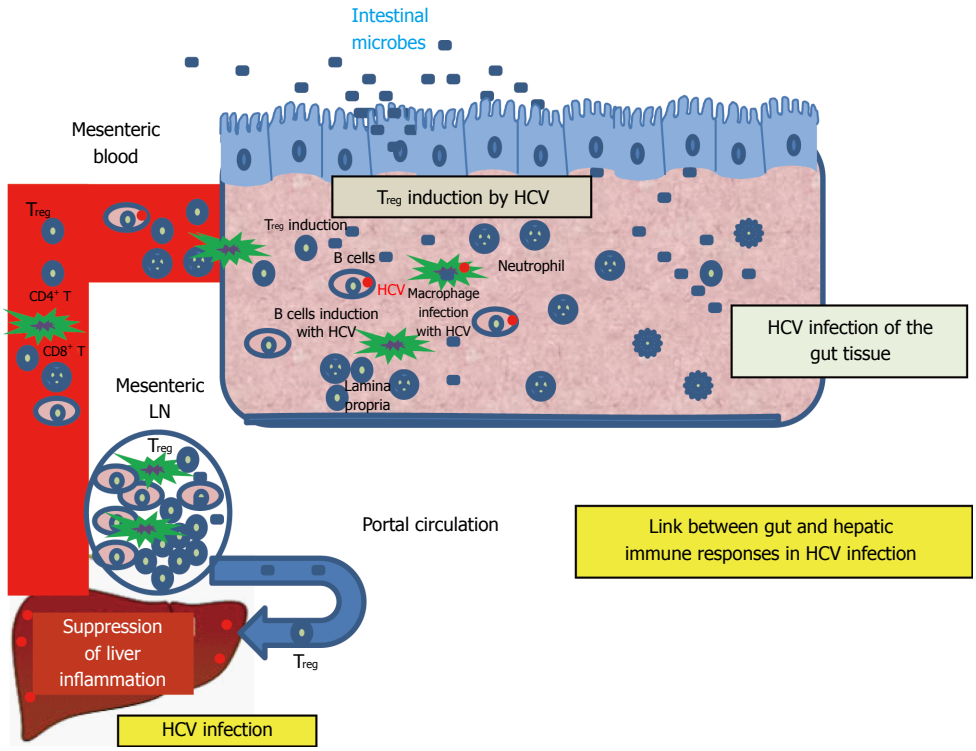


Figure 1 Link between Gut and hepatic immune responses in hepatitis C virus infection. Hepatitis C virus (HCV) replicates in the Gut B cells and macrophages and stimulates Treg cells. Colonic Treg cells migrate to the liver and inhibit immune responses to HCV infection, and inhibit liver inflammation and fibrosis.

T_H3 cell which are $CD4^+$ or $CD8^+$ T cells producing TGF-β with various amounts of interleukin-4 and interleukin-10[89]; and (3) T_{reg} cells, a population of naturally occurring $CD4^+CD25^+$ regulatory T cells that suppress proliferation through a cell contact-dependent mechanism[90] followed by cell-contact-independent mechanism mediated by soluble factors such as IL-10 and TGF-β[91]. Induction of tolerance is a contact-dependent mechanism used by naturally occurring $CD4^+CD25^+$ T_{reg} to confer suppressive activity upon conventional antigen-specific $CD4^+$ T cells through the expression of the transcription factor Foxp3 and/or the major histocompatibility complex (MHC) class II-binding molecule LAG-3 in such cells[69,91], and inhibit T cell activation *via* soluble mediators. $CD4^+CD25^+$ T_{reg} cells expressing the mucosal α4β7 integrin, when co-cultured with conventional $CD4^+$ T cells, induced Tr1-like IL-10-secreting T cells with strong suppressor activity on effector T cells. While α4β1-positive T_{reg} induced T_H3-like TGF-β secreting suppressor T cells[91]. Moreover, intraepithelial $CD8^+γδ$ T cells in the small intestine have been involved in mucosal tolerance and are the first T cells to encounter pathogens that have invaded an epithelial surface[92].

ROLE OF LIVER IN ORAL TOLERANCE

Although the liver is capable of generating vigorous immune responses to infections such as hepatitis A and hepatitis E viruses, both of which enter *via* the gut, it is also characterized by immune tolerance in several settings[93,94]. A vigorous intrahepatic immune response depends on activation of T cells by fully activated DCs within secondary lymphoid tissues whereas direct activation within the liver by resident APCs including endothelial cells and hepatocytes usually results in tolerance[95]. This is logic, as it allows the liver to tolerate soluble food antigens captured by liver endothelial cells and self-antigens on hepatocytes that fail to cause damage whilst responding appropriately to infections that cause injury, inflammation and full activation of DCs[67].

Regulatory T cells as well as NK and CD1-restricted NKT cells seem to contribute to the overall bias of hepatic immune responses toward tolerance. The tolerance microenvironment of the liver may account for the survival of liver allografts and the persistence of certain liver pathogens such as hepatitis viruses[94].

LINK BETWEEN THE GUT AND LIVER IMMUNE RESPONSES DURING HCV INFECTION

The Gut and the liver share common embryological origins; the liver develops from the ventral floor of the foregut as the liver diverticulum from the undifferentiated gut endoderm[96]. Subsequently, the gut is populated by lymphocyte precursors derived from the developing liver[97] (Figure 1).

There is an immunologic link between the gut and the liver through a population of T-cells that are capable of homing to both the liver and gut *via* portal circulation[96]. Additionally, the liver is considered an important

toleragenic organ for all of foreign proteins we are eating that are probably mediated through the T_{reg} cells, which in turn act as a link between the gut and the liver[67,96]. Most of the infiltrating T-cells in the liver are primed cells suggesting that trafficking of memory T-cells through the liver might contribute to immune surveillance[98]. Evidence, that supports such findings, comes from observations that the gut adhesion molecules and chemokine (such as CCL25) are also detected on liver endothelium[99] providing a mechanism for the recruitment of mucosal lymphocytes to the liver[100].

Evaluation of the gut immune cells for the intrinsic gut-liver immune axis of the shared lymphocytes that recirculate between the gut and liver through the portal circulation may be considered a useful image of the intrahepatic micro-environment during HCV infection. Based on this relationship, the frequency of T_{reg} cells in colonic tissue and its association with the various outcomes of anti-HCV therapy, viral persistence and degree of liver inflammation were examined in our laboratory. Our data indicated that the frequency of colonic T_{reg} in CHC patients is higher than control and our findings are in concordance with previous reports that demonstrated a higher number of $FoxP3^{+}T_{reg}$ cells in the liver of HCV-infected patients compared to healthy control[47,59,60]. These findings support that T_{reg} plays a prominent role in maintaining the balance between tissue damaging and protective effects of immune responses to HCV.

While attempting to limit viral replication, T-cells inadvertently play a pivotal role in limiting hepatic necroinflammation and subsequent fibrosis[28,101-103] by suppressing HCV-specific immune responses[48]. In our study, we found a significant inverse correlation between the frequency of colonic T_{reg} and liver pathology indicating a role of colonic T_{reg} in controlling the chronic inflammatory response and limit liver damage in CHC infection.

There is still an open question whether T_{reg} cells are protective or harmful in CHC. The effective host anti-HCV immune response may be associated with strong inflammatory reactions and liver damage. To minimize the damage to self, the activation of the immune system also triggers anti-inflammatory pathways through T_{reg} responses. Both inflammatory and anti-inflammatory reactions are normal components of the immune response, which together, fight infections while preventing immunopathology.

TREATMENT OF HCV AND RELATIONSHIP TO IMMUNE RESPONSES

Until 2011, the standard of care for chronic hepatitis C patients was combined treatment with Peginterferon (Peg-IFN) and ribavirin (RBV). The combination of Peg-IFN and RBV induced sustained virologic response (SVR) in 40%-50% of genotype 1 and 80% or more in genotype 2 and 3 infections[104-106]. The lack of effective regimens across all genotypes and alternative therapeutics for patients who suffered serious side effects prompted basic science research and numerous clinical trials leading to the development of direct-acting antiviral (DAA) agents. The US Food and Drug Administration approved Telaprevir (TVR) and Boceprevir (BOC) for HCV genotype 1. They inhibit HCV nonstructural protein 3/4A (NS3/4A) serine protease, which is critical for HCV replication. TVR and BOC are approved for use in combination therapies with Peg-IFN-alpha and RBV as they improved SVR rates to 75% and 66% respectively for adult HCV genotype 1 patients with compensated liver cirrhosis[107]. However, these DAAs incur their own set of severe side effects including anemia, rash, and hyperbilirubinemia. New drugs classified as second-wave protease inhibitors, second-generation protease inhibitors, and polymerase inhibitors are being developed and currently undergoing clinical trials[108]. The NS5B polymerase inhibitor, sofosbuvir has been recently approved by the FDA for treatment of hepatitis C genotype 1, 2, and 3 patients[109].

Identifying patients that are likely to achieve SVR versus those that are likely to be non-responders is crucial for disease prognosis, providing optimal therapy, avoiding side effects, and reducing costs associated with Hepatitis C therapy. Since sequencing of the human genome in 2001, advancements along with decrease costs in genotyping technologies have led to investigation of genomic markers associated with a response to Peg-IFN and RBV in patients with chronic hepatitis C. The rs12979860 SNP located on chromosome 19 upstream of the *IL-28B* gene has been identified as a significant predictor of SVR in HCV Genotype 1 chronically infected patients that underwent standard therapy[110]. The same rs12979860 SNP has the ability to predict natural clearance of the hepatitis C virus[30]. Genotype C/C at the rs12979860 SNP was associated with a higher likelihood of natural clearance and therapy induced clearance of hepatitis C genotype 1, while T/T genotype was the most unfavorable[111]. Studies have confirmed that rs12979860 is the strongest predictor of SVR and can effectively predict response to IFN/RBV based therapy[112]. The mechanisms by which the rs12979860 affects HCV pathogenesis are still unclear. However, it is well-known that the IL-28B gene codes for cytokine IL-28B also known as interferon (IFN) λ -3, which belongs to the type Ⅲ IFN family. IFN-λ is mainly produced by macrophages and DCs in response to viral proteins and plays an important role in antiviral responses to hepatitis C[30,113]. IFN λ receptors are predominantly expressed on hepatocytes, which may explain its ability to counteract hepatotropic viruses[114]. Therefore, stimulation of IFN λ receptors on hepatocytes by IFN-λ secreted by DCs induces ISGs[115] which have the ability to suppress viral replication and protein synthesis of HCV[116]. Additionally, IFN-λ promotes differentiation of monocyte-derived dendritic cells (DCs) with high PD-L1 expression and further promoted expansion of T_{reg} cells[117] locally and suppressed the inflammatory responses in the liver. Recent data by our laboratory (Hetta *et al*, 2014 submitted) as well as others[118] identified a correlation between IL28B SNP rs12979860 genotype TT'

s and T_{reg} frequencies. The mechanism responsible for elevated T_{reg} in patients with TT genotype may be related to the precise location of rs12979860 in the promoter region of the *IL-28B* gene. The promoter region plays an important role in gene expression, and the TT genotype might favor increased IL-28B expression in turn resulting in higher T_{reg} frequencies. In support of the relationship between IL-28B phenotypes, T_{reg} frequency, and HCV pathogenesis, recent reports found elevated T_{reg} in acute HCV as a predictor for viral persistence and CHC as well as increased levels of IFN-λ, IL-28, and IL-29 in serum in chronic HCV patients[117].

The association between IL-28B polymorphism and SVR in genotype 2 and 3 infected patients has produced mixed results making its clinical utility less clear. For instance, one study found IL-28B polymorphism to be associated with SVR in patients infected by genotype 2/3 HCV in whom RVR was not achieved[119]. On the other hand, in a study of hepatitis C Genotype 3 infected patients, rs12979860 SNP genotype C/C did not correlate with SVR to PEG-IFN/ribavirin therapy[120]. The majority of studies to this point have focused on IL-28B SNPs in HCV Genotype 1, 2, and 3. The clinical utility of IL-28B testing is probably best served in HCV genotype 1 infected-patients for prediction of outcomes and to limit expenses and side effects associated with IFN-based therapy[110].

REFERENCES

1 **Alter HJ**, Purcell RH, Holland PV, Popper H. Transmissible agent in non-A, non-B hepatitis. *Lancet* 1978; **1**: 459-463 [PMID: 76017 DOI: 10.1016/S0140-6736(78)90131-9]

2 **Wang KS**, Choo QL, Weiner AJ, Ou JH, Najarian RC, Thayer RM, Mullenbach GT, Denniston KJ, Gerin JL, Houghton M. Structure, sequence and expression of the hepatitis delta (delta) viral genome. *Nature* 1986; **323**: 508-514 [PMID: 3762705]

3 **Revie D**, Salahuddin SZ. Human cell types important for hepatitis C virus replication in vivo and in vitro: old assertions and current evidence. *Virol J* 2011; **8**: 346 [PMID: 21745397 DOI: 10.1186/1743-422x-8-346]

4 **Simmonds P**, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, Halfon P, Inchauspé G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin-I T, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 2005; **42**: 962-973 [PMID: 16149085 DOI: 10.1002/hep.20819]

5 **Farci P**, Purcell RH. Clinical significance of hepatitis C virus genotypes and quasispecies. *Semin Liver Dis* 2000; **20**: 103-126 [PMID: 10895435]

6 **Lauer GM**, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41-52 [PMID: 11439948 DOI: 10.1056/nejm200107053450107]

7 **Gouda I**, Nada O, Ezzat S, Eldaly M, Loffredo C, Taylor C, Abdel-Hamid M. Immunohistochemical detection of hepatitis C virus (genotype 4) in B-cell NHL in an Egyptian population: correlation with serum HCV-RNA. *Appl Immunohistochem Mol Morphol* 2010; **18**: 29-34 [PMID: 19644357 DOI: 10.1097/PAI.0b013e3181ae9e82]

8 **Frank C**, Mohamed MK, Strickland GT, Lavanchy D, Arthur RR, Magder LS, El Khoby T, Abdel-Wahab Y, Aly Ohn ES, Anwar W, Sallam I. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. *Lancet* 2000; **355**: 887-891 [PMID: 10752705 DOI: 10.1016/S0140-6736(99)06527-7]

9 **Klenerman P**, Thimme R. T cell responses in hepatitis C: the good, the bad and the unconventional. *Gut* 2012; **61**: 1226-1234 [PMID: 21873736 DOI: 10.1136/gutjnl-2011-30062]

10 **Kuo G**, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* 1989; **244**: 362-364 [PMID: 2496467 DOI: 10.1126/science.2496467]

11 **Alipour A**, Rezaianzadeh A, Hasanzadeh J, Rajaeefard A, Davarpanah MA. Sexual Transmission of Hepatitis C Virus Between HIV Infected Subjects and Their Main Heterosexual Partners. *Hepat Mon* 2013; **13**: e13593 [PMID: 24348647 DOI: 10.5812/hepatmon.13593]

12 **Mast EE**, Alter MJ, Margolis HS. Strategies to prevent and control hepatitis B and C virus infections: a global perspective. *Vaccine* 1999; **17**: 1730-1733 [PMID: 10194830 DOI: 10.1016/S0264-410X(98)00415-0]

13 EASL International Consensus Conference on hepatitis C. Paris, 26-27 February 1999. Consensus statement. *J Hepatol* 1999; **31** Suppl 1: 3-8 [PMID: 10622553]

14 **Griffin SD**, Beales LP, Clarke DS, Worsfold O, Evans SD, Jaeger J, Harris MP, Rowlands DJ. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett* 2003; **535**: 34-38 [PMID: 12560074 DOI: 10.1016/S0014-5793(02)03851-6]

15 **Castillo I**, Rodríguez-Iñigo E, Bartolomé J, de Lucas S, Ortíz-Movilla N, López-Alcorocho JM, Pardo M, Carreño V. Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. *Gut* 2005; **54**: 682-685 [PMID: 15831916 DOI: 10.1136/gut.2004.057281]

16 **Blackard JT**, Kemmer N, Sherman KE. Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences. *Hepatology* 2006; **44**: 15-22 [PMID: 16799966 DOI: 10.1002/hep.21283]

17 **Agnello V**, De Rosa FG. Extrahepatic disease manifestations of HCV infection: some current issues. *J Hepatol* 2004; **40**: 341-352 [PMID: 14739110 DOI: 10.1016/j.jhep.2003.10.009]

18 **Manzin A**, Candela M, Paolucci S, Caniglia ML, Gabrielli A, Clementi M. Presence of hepatitis C virus (HCV) genomic RNA and viral replicative intermediates in bone marrow and peripheral blood mononuclear cells from HCV-infected patients. *Clin Diagn Lab Immunol* 1994; **1**: 160-163 [PMID: 7496938]

19 **Wang JT**, Sheu JC, Lin JT, Wang TH, Chen DS. Detection of replicative form of hepatitis C virus RNA in peripheral blood mononuclear cells. *J Infect Dis* 1992; **166**: 1167-1169 [PMID: 1328405 DOI: 10.1093/infdis/166.5.1167]

20 **Chang TT**, Young KC, Yang YJ, Lei HY, Wu HL. Hepatitis C virus RNA in peripheral blood mononuclear cells: comparing acute and chronic hepatitis C virus infection. *Hepatology* 1996; **23**: 977-981 [PMID: 8621178 DOI: 10.1002/hep.510230506]

21 **Saleh MG**, Tibbs CJ, Koskinas J, Pereira LM, Bomford AB, Portmann BC, McFarlane IG, Williams R. Hepatic and extrahepatic hepatitis C virus replication in relation to response to interferon therapy. *Hepatology* 1994; **20**: 1399-1404 [PMID: 7982638 DOI: 10.1002/hep.1840200604]

22 **Yan FM**, Chen AS, Hao F, Zhao XP, Gu CH, Zhao LB, Yang DL, Hao LJ. Hepatitis C virus may infect extrahepatic tissues in patients with hepatitis C. *World J Gastroenterol* 2000; **6**: 805-811 [PMID: 11819700]

23 **Carrozzo M**, Quadri R, Latorre P, Pentenero M, Paganin S, Bertolusso G, Gandolfo S, Negro F. Molecular evidence that the hepatitis C virus replicates in the oral mucosa. *J Hepatol* 2002; **37**: 364-369 [PMID: 12175632 DOI: 10.1016/S0168-8278(02)00183-6]

24 **Féray C**, Samuel D, Thiers V, Gigou M, Pichon F, Bismuth A, Reynes M, Maisonneuve P, Bismuth H, Bréchot C. Reinfection of liver graft by hepatitis C virus after liver transplantation. *J Clin Invest* 1992; **89**: 1361-1365 [PMID: 1313453 DOI: 10.1172/jci115723]

25 **Tursi A**, Brandimante G, Chiarelli F, Spagnoli A, Torello M. Detection of HCV RNA in gastric mucosa-associated lymphoid tissue by in situ hybridization: evidence of a new extrahepatic localization of HCV with increased risk of gastric malt lymphoma. *Am J Gastroenterol* 2002; **97**: 1802-1806 [PMID: 12135039 DOI: 10.1111/j.1572-0241.2002.05848.x]

26 **Miglioresi L**, Riva E, Antonelli G, Russo F, Ricci GL. Localization of hepatitis C virus in gastrointestinal mucosa: a possible reservoir for relapse. *Hepatology* 2003; **38**: 775 [PMID: 12939605 DOI: 10.1053/jhep.2003.50322]

27 **McHutchison JG**, Poynard T, Esteban-Mur R, Davis GL, Goodman ZD, Harvey J, Ling MH, Garaud JJ, Albrecht JK, Patel K, Dienstag JL, Morgan T. Hepatic HCV RNA before and after treatment with interferon alone or combined with ribavirin. *Hepatology* 2002; **35**: 688-693 [PMID: 11870385 DOI: 10.1053/jhep.2002.31870]

28 **Rehermann B**. Interaction between the hepatitis C virus and the immune system. *Semin Liver Dis* 2000; **20**: 127-141 [PMID: 10946419 DOI: 10.1055/s-2000-9946]

29 **Kelly C**, Klenerman P, Barnes E. Interferon lambdas: the next cytokine storm. *Gut* 2011; **60**: 1284-1293 [PMID: 21303914 DOI: 10.1136/gut.2010.222976]

30 **Thomas DL**, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB, Carrington M. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; **461**: 798-801 [PMID: 19759533 DOI: 10.1038/nature08463]

31 **Rehermann B**. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. *J Clin Invest* 2009; **119**: 1745-1754 [PMID: 19587449 DOI: 10.1172/jci39133]

32 **Walker CM**. Adaptive immunity to the hepatitis C virus. *Adv Virus Res* 2010; **78**: 43-86 [PMID: 21040831 DOI: 10.1016/b978-0-12-385032-4.00002-1]

33 **Pestka JM**, Zeisel MB, Bläser E, Schürmann P, Bartosch B, Cosset FL, Patel AH, Meisel H, Baumert J, Viazov S, Rispeter K, Blum HE, Roggendorf M, Baumert TF. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci USA* 2007; **104**: 6025-6030 [PMID: 17392433 DOI: 10.1073/pnas.0607026104]

34 **Spengler U**, Nattermann J. Immunopathogenesis in hepatitis C virus cirrhosis. *Clin Sci* (Lond) 2007; **112**: 141-155 [PMID: 17199558 DOI: 10.1042/cs20060171]

35 **Post JJ**, Pan Y, Freeman AJ, Harvey CE, White PA, Palladinetti P, Haber PS, Marinos G, Levy MH, Kaldor JM, Dolan KA, Ffrench RA, Lloyd AR, Rawlinson WD. Clearance of hepatitis C viremia associated with cellular immunity in the absence of seroconversion in the hepatitis C incidence and transmission in prisons study cohort. *J Infect Dis* 2004; **189**: 1846-1855 [PMID: 15122521 DOI: 10.1086/383279]

36 **Lucas M**, Ulsenheimer A, Pfafferot K, Heeg MH, Gaudieri S, Grüner N, Rauch A, Gerlach JT, Jung MC, Zachoval R, Pape GR, Schraut W, Santantonio T, Nitschko H, Obermeier M, Phillips R, Scriba TJ, Semmo N, Day C, Weber JN, Fidler S, Thimme R, Haberstroh A, Baumert TF, Klenerman P, Diepolder HM. Tracking virus-specific CD4+ T cells during and after acute hepatitis C virus infection. *PLoS One* 2007; **2**: e649 [PMID: 17653276 DOI: 10.1371/journal.pone.0000649]

37 **Missale G**, Bertoni R, Lamonaca V, Valli A, Massari M, Mori C, Rumi MG, Houghton M, Fiaccadori F, Ferrari C. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. *J Clin Invest* 1996; **98**: 706-714 [PMID: 8698862 DOI: 10.1172/jci118842]

38 **Grakoui A**, Shoukry NH, Woollard DJ, Han JH, Hanson HL, Ghrayeb J, Murthy KK, Rice CM, Walker CM. HCV persistence and immune evasion in the absence of memory T cell help. *Science* 2003; **302**: 659-662 [PMID: 14576438 DOI: 10.1126/science.1088774]

39 **Gruener NH**, Lechner F, Jung MC, Diepolder H, Gerlach T, Lauer G, Walker B, Sullivan J, Phillips R, Pape GR, Klenerman P. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J Virol* 2001; **75**: 5550-5558 [PMID: 11356962 DOI: 10.1128/jvi.75.12.5550-5558.2001]

40 **Nakamoto N**, Kaplan DE, Coleclough J, Li Y, Valiga ME, Kaminski M, Shaked A, Olthoff K, Gostick E, Price DA, Freeman GJ, Wherry EJ, Chang KM. Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. *Gastroenterology* 2008; **134**: 1927-1937, 1937.e1-2 [PMID: 18549878 DOI: 10.1053/j.gastro.2008.02.033]

41 **Spangenberg HC**, Viazov S, Kersting N, Neumann-Haefelin C, McKinney D, Roggendorf M, von Weizsäcker F, Blum HE, Thimme R. Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. *Hepatology* 2005; **42**: 828-837 [PMID: 16175596 DOI: 10.1002/hep.20856]

42 **McMahan RH**, Golden-Mason L, Nishimura MI, McMahon BJ, Kemper M, Allen TM, Gretch DR, Rosen HR. Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *J Clin Invest* 2010; **120**: 4546-4557 [PMID: 21084749 DOI: 10.1172/jci43127]

43 **Radziewicz H**, Ibegbu CC, Fernandez ML, Workowski KA, Obideen K, Wehbi M, Hanson HL, Steinberg JP, Masopust D, Wherry EJ, Altman JD, Rouse BT, Freeman GJ, Ahmed R, Grakoui A. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol* 2007; **81**: 2545-2553 [PMID: 17182670 DOI: 10.1128/jvi.02021-06]

44 **Bengsch B**, Seigel B, Ruhl M, Timm J, Kuntz M, Blum HE, Pircher H, Thimme R. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathog* 2010; **6**: e1000947 [PMID: 20548953 DOI: 10.1371/journal.ppat.1000947]

45 **Rutebemberwa A**, Ray SC, Astemborski J, Levine J, Liu L, Dowd KA, Clute S, Wang C, Korman A, Sette A, Sidney J, Pardoll DM, Cox AL. High-programmed death-1 levels on hepatitis C virus-specific T cells during acute infection are associated with viral persistence and require preservation of cognate antigen during chronic infection. *J Immunol* 2008; **181**: 8215-8225 [PMID: 19050238 DOI: 10.4049/jimmunol.181.12.8215]

46 **Thimme R**, Opitz OG. Interleukin-10 and viral clearance: translation to viral hepatitis. *Gastroenterology* 2007; **132**: 2611-2613 [PMID: 17570238 DOI: 10.1053/j.gastro.2007.04.049]

47 **Boettler T**, Spangenberg HC, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, Blum HE, von Weizsäcker F, Thimme R. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol* 2005; **79**: 7860-7867 [PMID: 15919940 DOI: 10.1128/jvi.79.12.7860-7867.2005]

48 **Cabrera R**, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, Nelson DR. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 2004; **40**: 1062-1071 [PMID: 15486925 DOI: 10.1002/hep.20454]

49 **Amoroso A**, D'Amico F, Consolo M, Skarmoutsou E, Neri S, Dianzani U, Spandidos DA, Mazzarino MC. Evaluation of circulating CD4+CD25+ and liver-infiltrating Foxp3+ cells in HCV-associated liver disease. *Int J Mol Med* 2012; **29**: 983-988

[PMID: 22446965 DOI: 10.3892/ijmm.2012.947]
50 **Sugimoto K**, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* 2003; **38**: 1437-1448 [PMID: 14647055 DOI: 10.1016/j.hep.2003.09.026]
51 **Ward SM**, Fox BC, Brown PJ, Worthington J, Fox SB, Chapman RW, Fleming KA, Banham AH, Klenerman P. Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection. *J Hepatol* 2007; **47**: 316-324 [PMID: 17475362 DOI: 10.1016/j.jhep.2007.03.023]
52 **Bowen DG**, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 2005; **436**: 946-952 [PMID: 16107834 DOI: 10.1038/nature04079]
53 **Keynan Y**, Card CM, McLaren PJ, Dawood MR, Kasper K, Fowke KR. The role of regulatory T cells in chronic and acute viral infections. *Clin Infect Dis* 2008; **46**: 1046-1052 [PMID: 18444822 DOI: 10.1086/529379]
54 **Hartling HJ**, Gaardbo JC, Ronit A, Knudsen LS, Ullum H, Vainer B, Clausen MR, Skogstrand K, Gerstoft J, Nielsen SD. $CD4^+$ and $CD8^+$ regulatory T cells (Tregs) are elevated and display an active phenotype in patients with chronic HCV mono-infection and HIV/HCV co-infection. *Scand J Immunol* 2012; **76**: 294-305 [PMID: 22671952 DOI: 10.1111/j.1365-3083.2012.02725.x]
55 **Bolacchi F**, Sinistro A, Ciaprini C, Demin F, Capozzi M, Carducci FC, Drapeau CM, Rocchi G, Bergamini A. Increased hepatitis C virus (HCV)-specific CD4+CD25+ regulatory T lymphocytes and reduced HCV-specific CD4+ T cell response in HCV-infected patients with normal versus abnormal alanine aminotransferase levels. *Clin Exp Immunol* 2006; **144**: 188-196 [PMID: 16634790]
56 **Sturm N**, Thélu MA, Camous X, Dimitrov G, Ramzan M, Dufeu-Duchesne T, Bonorino P, Guillermet C, Brambilla E, Arvers P, Pernollet M, Leroy V, Zarski JP, Marche PN, Jouvin-Marche E. Characterization and role of intra-hepatic regulatory T cells in chronic hepatitis C pathogenesis. *J Hepatol* 2010; **53**: 25-35 [PMID: 20452085 DOI: 10.1016/j.jhep.2010.02.024]
57 **Sakaguchi S**, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008; **133**: 775-787 [PMID: 18510923 DOI: 10.1016/j.cell.2008.05.009]
58 **Magg T**, Mannert J, Ellwart JW, Schmid I, Albert MH. Subcellular localization of FOXP3 in human regulatory and non-regulatory T cells. *Eur J Immunol* 2012; **42**: 1627-1638 [PMID: 22678915 DOI: 10.1002/eji.201141838]
59 **Rushbrook SM**, Ward SM, Unitt E, Vowler SL, Lucas M, Klenerman P, Alexander GJ. Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. *J Virol* 2005; **79**: 7852-7859 [PMID: 15919939 DOI: 10.1128/jvi.79.12.7852-7859.2005]
60 **Itose I**, Kanto T, Kakita N, Takebe S, Inoue M, Higashitani K, Miyazaki M, Miyatake H, Sakakibara M, Hiramatsu N, Takehara T, Kasahara A, Hayashi N. Enhanced ability of regulatory T cells in chronic hepatitis C patients with persistently normal alanine aminotransferase levels than those with active hepatitis. *J Viral Hepat* 2009; **16**: 844-852 [PMID: 19486278 DOI: 10.1111/j.1365-2893.2009.01131.x]
61 **Brandtzaeg P**. Mucosal immunity: induction, dissemination, and effector functions. *Scand J Immunol* 2009; **70**: 505-515 [PMID: 19906191 DOI: 10.1111/j.1365-3083.2009.02319.x]
62 **Brandtzaeg P**. History of oral tolerance and mucosal immunity. *Ann N Y Acad Sci* 1996; **778**: 1-27 [PMID: 8610963 DOI: 10.1111/j.1749-6632.1996.tb21110.x]
63 **Brandtzaeg P**. 'ABC' of mucosal immunology. *Nestle Nutr Workshop Ser Pediatr Program* 2009; **64**: 23-38; discussion 38-43, 251-257 [PMID: 19710513 DOI: 10.1159/000235781]
64 **Artis D**. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev Immunol* 2008; **8**: 411-420 [PMID: 18469830 DOI: 10.1038/nri2316]
65 **Husby S**, Jensenius JC, Svehag SE. Passage of undegraded dietary antigen into the blood of healthy adults. Quantification, estimation of size distribution, and relation of uptake to levels of specific antibodies. *Scand J Immunol* 1985; **22**: 83-92 [PMID: 4023632 DOI: 10.1111/j.1365-3083.1985.tb01862.x]
66 **Limmer A**, Ohl J, Wingender G, Berg M, Jüngerkes F, Schumak B, Djandji D, Scholz K, Klevenz A, Hegenbarth S, Momburg F, Hämmerling GJ, Arnold B, Knolle PA. Cross-presentation of oral antigens by liver sinusoidal endothelial cells leads to CD8 T cell tolerance. *Eur J Immunol* 2005; **35**: 2970-2981 [PMID: 16163670 DOI: 10.1002/eji.200526034]
67 **Adams DH**, Eksteen B, Curbishley SM. Immunology of the gut and liver: a love/hate relationship. *Gut* 2008; **57**: 838-848 [PMID: 18203807 DOI: 10.1136/gut.2007.122168]
68 **Yang R**, Liu Q, Grosfeld JL, Pescovitz MD. Intestinal venous drainage through the liver is a prerequisite for oral tolerance induction. *J Pediatr Surg* 1994; **29**: 1145-1148 [PMID: 7965523 DOI: 10.1016/0022-3468(94)90297-6]
69 **Holmgren J**, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med* 2005; **11**: S45-S53 [PMID: 15812489 DOI: 10.1038/nm1213]
70 **Agace WW**. Tissue-tropic effector T cells: generation and targeting opportunities. *Nat Rev Immunol* 2006; **6**: 682-692 [PMID: 16932753 DOI: 10.1038/nri1869]
71 **Shata MT**, Abdel-Hameed EA, Hetta HF, Sherman KE. Immune activation in HIV/HCV-infected patients is associated with low-level expression of liver expressed antimicrobial peptide-2 (LEAP-2). *J Clin Pathol* 2013; **66**: 967-975 [PMID: 23940131 DOI: 10.1136/jclinpath-2013-201581]
72 **Yuan Q**, Walker WA. Innate immunity of the gut: mucosal defense in health and disease. *J Pediatr Gastroenterol Nutr* 2004; **38**: 463-473 [PMID: 15097431 DOI: 10.1097/00005176-200405000-00001]
73 **Heremans JF**, Bazin H. Antibodies induced by local antigenic stimulation of mucosal surfaces. *Ann N Y Acad Sci* 1971; **190**: 268-275 [PMID: 5290019 DOI: 10.1111/j.1749-6632.1971.tb13540.x]
74 **Doe WF**. The intestinal immune system. *Gut* 1989; **30**: 1679-1685 [PMID: 2693229 DOI: 10.1136/gut.30.12.1679]
75 **Ogra PL**, Karzon DT. Poliovirus antibody response in serum and nasal secretions following intranasal inoculation with inactivated poliovaccine. *J Immunol* 1969; **102**: 15-23 [PMID: 4303877]
76 **Lycke N**, Eriksen L, Holmgren J. Protection against cholera toxin after oral immunization is thymus-dependent and associated with intestinal production of neutralizing IgA antitoxin. *Scand J Immunol* 1987; **25**: 413-419 [PMID: 3576135 DOI: 10.1111/j.1365-3083.1987.tb02208.x]
77 **Goodrich ME**, McGee DW. Regulation of mucosal B cell immunoglobulin secretion by intestinal epithelial cell-derived cytokines. *Cytokine* 1998; **10**: 948-955 [PMID: 10049518 DOI: 10.1006/cyto.1998.0385]
78 **Asano T**, Kaneko H, Terada T, Kasahara Y, Fukao T, Kasahara K, Kondo N. Molecular analysis of B-cell differentiation in selective or partial IgA deficiency. *Clin Exp Immunol* 2004; **136**: 284-290 [PMID: 15086392 DOI: 10.1111/j.1365-2249.2004.02440.x]
79 **Franco MA**, Greenberg HB. Role of B cells and cytotoxic T lymphocytes in clearance of and immunity to rotavirus infection in mice. *J Virol* 1995; **69**: 7800-7806 [PMID: 7494291]
80 **Buzoni-Gatel D**, Lepage AC, Dimier-Poisson IH, Bout DT, Kasper LH. Adoptive transfer of gut intraepithelial lymphocytes protects against murine infection with Toxoplasma gondii. *J Immunol* 1997; **158**: 5883-5889 [PMID: 9190941]
81 **Iwasaki A**, Kelsall BL. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J Exp Med* 1999; **190**: 229-239 [PMID: 10432286 DOI: 10.1084/jem.190.2.229]

82 **Rossi M**, Bot A. The Th17 cell population and the immune homeostasis of the gastrointestinal tract. *Int Rev Immunol* 2013; **32**: 471-474 [PMID: 24164337 DOI: 10.3109/08830185.2013.843983]

83 **Bilsborough J**, Viney JL. Gastrointestinal dendritic cells play a role in immunity, tolerance, and disease. *Gastroenterology* 2004; **127**: 300-309 [PMID: 15236195 DOI: 10.1053/j.gastro.2004.01.028]

84 **Mowat AM**. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 2003; **3**: 331-341 [PMID: 12669023 DOI: 10.1038/nri1057]

85 **Faria AM**, Weiner HL. Oral tolerance. *Immunol Rev* 2005; **206**: 232-259 [PMID: 16048553 DOI: 10.1111/j.0105-2896.2005.00280.x]

86 **Whitacre CC**, Gienapp IE, Orosz CG, Bitar DM. Oral tolerance in experimental autoimmune encephalomyelitis. III. Evidence for clonal anergy. *J Immunol* 1991; **147**: 2155-2163 [PMID: 1717550]

87 **Chen Y**, Inobe J, Marks R, Gonnella P, Kuchroo VK, Weiner HL. Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature* 1995; **376**: 177-180 [PMID: 7603570 DOI: 10.1038/376177a0]

88 **Groux H**, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997; **389**: 737-742 [PMID: 9338786 DOI: 10.1038/39614]

89 **Chen Y**, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 1994; **265**: 1237-1240 [PMID: 7520605 DOI: 10.1126/science.7520605]

90 **Thornton AM**, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998; **188**: 287-296 [PMID: 9670041 DOI: 10.1084/jem.188.2.287]

91 **Stassen M**, Fondel S, Bopp T, Richter C, Müller C, Kubach J, Becker C, Knop J, Enk AH, Schmitt S, Schmitt E, Jonuleit H. Human CD25+ regulatory T cells: two subsets defined by the integrins alpha 4 beta 7 or alpha 4 beta 1 confer distinct suppressive properties upon CD4+ T helper cells. *Eur J Immunol* 2004; **34**: 1303-1311 [PMID: 15114663 DOI: 10.1002/eji.200324656]

92 **McMenamin C**, Pimm C, McKersey M, Holt PG. Regulation of IgE responses to inhaled antigen in mice by antigen-specific gamma delta T cells. *Science* 1994; **265**: 1869-1871 [PMID: 7916481 DOI: 10.1126/science.7916481]

93 **Knolle PA**, Limmer A. Neighborhood politics: the immunoregulatory function of organ-resident liver endothelial cells. *Trends Immunol* 2001; **22**: 432-437 [PMID: 11473832 DOI: 10.1016/S1471-4906(01)01957-3]

94 **Crispe IN**. Hepatic T cells and liver tolerance. *Nat Rev Immunol* 2003; **3**: 51-62 [PMID: 12511875 DOI: 10.1038/nri981]

95 **Bowen DG**, McCaughan GW, Bertolino P. Intrahepatic immunity: a tale of two sites? *Trends Immunol* 2005; **26**: 512-517 [PMID: 16109501 DOI: 10.1016/j.it.2005.08.005]

96 **Gualdi R**, Bossard P, Zheng M, Hamada Y, Coleman JR, Zaret KS. Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes Dev* 1996; **10**: 1670-1682 [PMID: 8682297 DOI: 10.1101/gad.10.13.1670]

97 **Yoshida H**, Kawamoto H, Santee SM, Hashi H, Honda K, Nishikawa S, Ware CF, Katsura Y, Nishikawa SI. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. *J Immunol* 2001; **167**: 2511-2521 [PMID: 11509590 DOI: 10.4049/jimmunol.167.5.2511]

98 **Ward SM**, Jonsson JR, Sierro S, Clouston AD, Lucas M, Vargas AL, Powell EE, Klenerman P. Virus-specific CD8+ T lymphocytes within the normal human liver. *Eur J Immunol* 2004; **34**: 1526-1531 [PMID: 15162421 DOI: 10.1002/eji.200324275]

99 **Grant AJ**, Lalor PF, Hübscher SG, Briskin M, Adams DH. MAdCAM-1 expressed in chronic inflammatory liver disease supports mucosal lymphocyte adhesion to hepatic endothelium (MAdCAM-1 in chronic inflammatory liver disease). *Hepatology* 2001; **33**: 1065-1072 [PMID: 11343233 DOI: 10.1053/jhep.2001.24231]

100 **Eksteen B**, Grant AJ, Miles A, Curbishley SM, Lalor PF, Hübscher SG, Briskin M, Salmon M, Adams DH. Hepatic endothelial CCL25 mediates the recruitment of CCR9+ gut-homing lymphocytes to the liver in primary sclerosing cholangitis. *J Exp Med* 2004; **200**: 1511-1517 [PMID: 15557349 DOI: 10.1084/jem.20041035]

101 **Napoli J**, Bishop GA, McGuinness PH, Painter DM, McCaughan GW. Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. *Hepatology* 1996; **24**: 759-765 [PMID: 8855173 DOI: 10.1002/hep.510240402]

102 **Quiroga JA**, Martín J, Navas S, Carreño V. Induction of interleukin-12 production in chronic hepatitis C virus infection correlates with the hepatocellular damage. *J Infect Dis* 1998; **178**: 247-251 [PMID: 9652448 DOI: 10.1086/517446]

103 **Abrignani S**. Immune responses throughout hepatitis C virus (HCV) infection: HCV from the immune system point of view. *Springer Semin Immunopathol* 1997; **19**: 47-55 [PMID: 9266630 DOI: 10.1007/BF00945024]

104 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982 [PMID: 12324553 DOI: 10.1056/NEJMoa020047]

105 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965 [PMID: 11583749 DOI: 10.1016/S0140-6736(01)06102-5]

106 **Hadziyannis SJ**, Sette H, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355 [PMID: 14996676 DOI: 10.7326/0003-4819-140-5-200403020-00010]

107 **Ghany MG**, Nelson DR, Strader DB, Thomas DL, Seeff LB. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. *Hepatology* 2011; **54**: 1433-1444 [PMID: 21898493 DOI: 10.1002/hep.24641]

108 **Chae HB**, Park SM, Youn SJ. Direct-acting antivirals for the treatment of chronic hepatitis C: open issues and future perspectives. *ScientificWorldJournal* 2013; **2013**: 704912 [PMID: 23844410 DOI: 10.1155/2013/704912]

109 **Koff RS**. Review article: the efficacy and safety of sofosbuvir, a novel, oral nucleotide NS5B polymerase inhibitor, in the treatment of chronic hepatitis C virus infection. *Aliment Pharmacol Ther* 2014; **39**: 478-487 [PMID: 24387618 DOI: 10.1111/apt.12601]

110 **Ge D**, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; **461**: 399-401 [PMID: 19684573 DOI: 10.1038/nature08309]

111 **Pearlman BL**. The IL-28 genotype: how it will affect the care of patients with hepatitis C virus infection. *Curr Gastroenterol Rep* 2011; **13**: 78-86 [PMID: 21080244 DOI: 10.1007/s11894-010-0161-9]

112 **Thompson AJ**, Muir AJ, Sulkowski MS, Ge D, Fellay J, Shianna KV, Urban T, Afdhal NH, Jacobson IM, Esteban R, Poordad F, Lawitz EJ, McCone J, Shiffman ML, Galler GW,

Lee WM, Reindollar R, King JW, Kwo PY, Ghalib RH, Freilich B, Nyberg LM, Zeuzem S, Poynard T, Vock DM, Pieper KS, Patel K, Tillmann HL, Noviello S, Koury K, Pedicone LD, Brass CA, Albrecht JK, Goldstein DB, McHutchison JG. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. *Gastroenterology* 2010; **139**: 120-129.e18 [PMID: 20399780 DOI: 10.1053/j.gastro.2010.04.013]

113 **Ank N**, Iversen MB, Bartholdy C, Staeheli P, Hartmann R, Jensen UB, Dagnaes-Hansen F, Thomsen AR, Chen Z, Haugen H, Klucher K, Paludan SR. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *J Immunol* 2008; **180**: 2474-2485 [PMID: 18250457 DOI: 10.4049/jimmunol.180.4.2474]

114 **Sheppard P**, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J, Ostrander C, Dong D, Shin J, Presnell S, Fox B, Haldeman B, Cooper E, Taft D, Gilbert T, Grant FJ, Tackett M, Krivan W, McKnight G, Clegg C, Foster D, Klucher KM. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 2003; **4**: 63-68 [PMID: 12469119 DOI: 10.1038/ni873]

115 **Stark GR**, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem* 1998; **67**: 227-264 [PMID: 9759489 DOI: 10.1146/annurev.biochem.67.1.227]

116 **Samuel CE**. Antiviral actions of interferons. *Clin Microbiol Rev* 2001; **14**: 778-809, table of contents [PMID: 11585785 DOI: 10.1128/CMR.14.4.778-809.2001]

117 **Dolganiuc A**, Kodys K, Marshall C, Saha B, Zhang S, Bala S, Szabo G. Type III interferons, IL-28 and IL-29, are increased in chronic HCV infection and induce myeloid dendritic cell-mediated FoxP3+ regulatory T cells. *PLoS One* 2012; **7**: e44915 [PMID: 23071503 DOI: 10.1371/journal.pone.0044915]

118 **Perrella A**, Vitiello L, Atripaldi L, Conti P, Sbreglia C, Altamura S, Patarino T, Vela R, Morelli G, Bellopede P, Alone C, Racioppi L, Perrella O. Elevated CD4+/CD25+ T cell frequency and function during acute hepatitis C presage chronic evolution. *Gut* 2006; **55**: 1370-1371 [PMID: 16905711 DOI: 10.1136/gut.2006.099887]

119 **Mangia A**, Thompson AJ, Santoro R, Piazzolla V, Tillmann HL, Patel K, Shianna KV, Mottola L, Petruzzellis D, Bacca D, Carretta V, Minerva N, Goldstein DB, McHutchison JG. An IL28B polymorphism determines treatment response of hepatitis C virus genotype 2 or 3 patients who do not achieve a rapid virologic response. *Gastroenterology* 2010; **139**: 821-827, 827.e1 [PMID: 20621700 DOI: 10.1053/j.gastro.2010.05.079]

120 **Moghaddam A**, Melum E, Reinton N, Ring-Larsen H, Verbaan H, Bjøro K, Dalgard O. IL28B genetic variation and treatment response in patients with hepatitis C virus genotype 3 infection. *Hepatology* 2011; **53**: 746-754 [PMID: 21374656 DOI: 10.1002/hep.24154]

P- Reviewer: Dirchwolf M, Narciso-Schiavon JL, Puoti C, Sagnelli E **S- Editor**: Song XX **L- Editor**: A **E- Editor**: Wang CH

World Journal of Immunology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5411/wji.v4.i2.63

World J Immunol 2014 July 27; 4(2): 63-77
ISSN 2219-2824 (online)

REVIEW

CD28/CTLA-4/B7 and CD40/CD40L costimulation and activation of regulatory T cells

Isabel T Vogel, Stefaan W Van Gool, Jan L Ceuppens

Isabel T Vogel, Stefaan W Van Gool, Jan L Ceuppens, Laboratory of Clinical Immunology, Department of Microbiology and Immunology, KU Leuven, University Hospital Gasthuisberg, 3000 Leuven, Belgium
Stefaan W Van Gool, Laboratory of Pediatric Immunology, KU Leuven, University Hospital Gasthuisberg, 3000 Leuven, Belgium
Author contributions: All authors contributed to this manuscript.
Correspondence to: Jan L Ceuppens, MD, Laboratory of Clinical Immunology, Department of Microbiology and Immunology, KU Leuven, University Hospital Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium. jan.ceuppens@uzleuven.be
Telephone: +32-16-346137 Fax: +32-16-346035
Received: March 27, 2014 Revised: May 12, 2014
Accepted: June 10, 2014
Published online: July 27, 2014

Abstract

Costimulatory signals are crucial for T cell activation. Attempts to block costimulatory pathways have been effective in preventing unwanted immune reactions. In particular, blocking the CD28/cytotoxic T lymphocyte antigen (CTLA)-4/B7 interaction (using CTLA-4Ig) and the CD40/CD40L interaction (using anti-CD40L antibodies) prevents T cell mediated autoimmune diseases, transplant rejection and graft *vs* host disease in experimental models. Moreover, CTLA-4Ig is in clinical use to treat rheumatoid arthritis (abatacept) and to prevent rejection of renal transplants (belatacept). Under certain experimental conditions, this treatment can even result in tolerance. Surprisingly, the underlying mechanisms of immune modulation are still not completely understood. We here discuss the evidence that costimulation blockade differentially affects effector T cells (Teff) and regulatory T cells (Treg). The latter are required to control inappropriate and unwanted immune responses, and their activity often contributes to tolerance induction and maintenance. Unfortunately, our knowledge on the costimulatory requirements of Treg cells is very limited. We therefore summarize the current understanding of the costimulatory requirements of Treg cells, and elaborate on the effect of anti-CD40L antibody and CTLA-4Ig treatment on Treg cell activity. In this context, we point out that the outcome of a treatment aiming at blocking the CD28/CTLA-4/B7 costimulatory interaction can vary with dosing, timing and underlying immunopathology.

Key words: Regulatory T cells; Tolerance; Cytotoxic T lymphocyte antigen-4Ig; Anti-CD40L; Costimulation

Core tip: Costimulation blockade (*e.g.*, CD28/B7 and CD40/CD40L blockade) has been successfully used experimentally to induce tolerance to allo- or autoantigens. Several studies suggest that effector T cells (Teff) and regulatory T cells (Treg) have different requirements regarding costimulation. While blockade of the CD40L receptor does not affect Treg cells and targets Teff cells, the effect of blocking the CD28/cytotoxic T lymphocyte antigen (CTLA)-4/B7 interaction (with CTLA-4Ig) is more difficult to predict and depends on the type, the strength and the stage of an immune process. Importantly, manipulating these costimulatory signals can therefore shift the Treg/Teff cell balance towards dominant Treg cell activity.

Vogel IT, Van Gool SW, Ceuppens JL. CD28/CTLA-4/B7 and CD40/CD40L costimulation and activation of regulatory T cells. *World J Immunol* 2014; 4(2): 63-77 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i2/63.htm DOI: http://dx.doi.org/10.5411/wji.v4.i2.63

INTRODUCTION

Costimulatory interactions between T cells and antigen presenting cells (APCs), such as the CD28/B7 pathway

and the CD40/CD40L pathway, are essential for T cell activation. As a consequence, reagents that deliberately block those costimulatory signals (*e.g.*, the CTLA-4Ig fusion protein or antagonistic anti-CD40L antibodies) can be used to prevent unwanted or inappropriate T cell activation. Blocking costimulation, therefore, has been used to treat T cell mediated autoimmune diseases, transplant rejection or graft *vs* host disease (GvHD). Although anti-CD40L antibodies showed great potential in pre-clinical animal models and cytotoxic T lymphocyte antigen (CTLA)-4Ig is successfully used in clinical practice to treat rheumatoid arthritis and to prevent rejection of renal transplants, the precise mechanisms underlying their efficacy are still not fully understood. While effector T cells (Teff) clearly depend on costimulation for their activation, the costimulatory requirements of a suppressive T cell population, the regulatory T cells (Treg), are not completely clear. Several studies suggest that Treg and Teff cells have different requirements regarding costimulation. Furthermore, it has been suggested that Treg cells play an important role in the process of tolerance induction by costimulation blockade. In this review we discuss some possibilities to modulate costimulation in such a way that Teff cells are blocked but Treg cells remain active and functional. In this context, we summarize the current understanding of the costimulatory requirements of Treg cells, and elaborate on the effect of anti-CD40L antibody and CTLA-4Ig treatment on Treg cells. We point out that CTLA-4Ig has a quite complex effect on Treg cells, which should be taken into account when interfering with the CD28/CTLA-4/B7 interaction.

MECHANISMS OF PERIPHERAL TOLERANCE

Immune tolerance refers to a state of specific immune non-responsiveness of the immune system to a particular antigen or a group of antigens. Tolerance to self-antigens is a hallmark of an effectively functioning immune system and disabling tolerance to self-antigens can lead to autoimmune diseases. In a similar way, an inappropriate response to a harmless environmental antigen can result in allergies. To avoid such harmful reactions, the immune system has developed several sophisticated mechanisms to induce and maintain tolerance.

During the maturation in the thymus, T cells undergo positive and negative selection. T cells which recognize a self-antigen presented by major histocompatibility complex (MHC) molecules, can be eliminated (negative selection)[1]. In this process, the signal strength with which the T cell receptor (TCR) recognizes its antigen determines the fate of the T cell. A strong signal and definite recognition of the auto-antigen leads to immediate deletion of the responding cell. A weak signal often leads to ignorance and migration to the periphery[2]. This is reasonable in order to maintain a pool of variable TCRs in the periphery. However, these cells might regain self-reactivity later on. Furthermore, some T cells escape thymic selection. Under these circumstances, peripheral tolerance induction should come into action.

Peripheral tolerance is maintained by mechanisms such as anergy (which results from a lack of sufficient activation signals)[3], deletion by apoptosis[4,5] and control by regulatory T (Treg) cells. The role of regulatory T cells, as well as the importance of costimulation for the induction and maintenance of peripheral tolerance, will be discussed in the following section.

Costimulatory signals

Naïve T cells need two distinct signals in order to get fully activated[6]. The first signal is transmitted through the TCR, which recognizes an antigen presented by specialized antigen-presenting cells (APCs) on MHC molecules. This signal determines the specificity of the T cell response. The second (or accessory) signal is provided by the ligation of costimulatory receptors on the cell surface[7]. Without proper costimulation, T cells fail to become fully activated and enter a state of hypo-responsiveness (anergy)[8]. Up to now, many costimulatory signals and pathways have been identified, among which the best characterized are the CD28/CTLA-4/B7 pathway and the CD40/CD40L pathway.

The CD28/CTLA-4/B7 interaction: Mice deficient in CD28 are unable to mount an effective immune response to foreign antigens, pathogens or allografts. The CD28 receptor is a disulfide-linked homodimer, which is constitutively expressed on T cells and is engaged by both the CD80 (B7-1) and CD86 (B7-2) molecule on activated APC[9]. The monomeric CD86 ligand is constitutively expressed in low amounts on professional APC and up-regulated upon activation, while CD80 is expressed as a dimer on activated APC. The up-regulation of CD86 occurs rapidly after activation and reaches its maximum 18 to 24 h after stimulation, while the up-regulation of CD80 is delayed and reaches a maximum after 48 to 72 h[10,11]. Studies with knock-out (KO) mice have shown that CD86 is more important for initiating an immune response than CD80. Otherwise the functions of the two B7 molecules are largely overlapping[12]. Signalling *via* CD28 is mediated through the phosphatidylinositol 3-kinase-protein kinase B (PKB/Akt) and the growth factor-receptor-bound protein 2 (Grb2) pathways and promotes IL-2 production[13] and T cell proliferation[14] by decreasing the threshold for activation *via* the TCR[15]. In addition, T cell survival is strengthened by up-regulation of the anti-apoptotic factor Bcl-xL[16]. CD28 engagement also up-regulates or induces the expression of additional costimulatory receptors such as ICOS and CTLA-4[17]. While CD28/B7 signalling is crucial for the activation of naïve T cells, previously activated cells are less dependent on costimulation. After priming and differentiation are completed, the production of effector cytokines (*e.g.*, IL-4 or IFNγ) does not require further costimulation. Only IL-2 production depends on continuous costimulatory signalling[12].

Another receptor molecule, which binds to both B7 molecules and is structurally homologous to CD28, is the "cytotoxic T lymphocyte antigen 4" (CTLA-4) or CD152. It is up-regulated on T cells upon activation with a peak at 24-48 h after initial priming[18]. However, its expression on the surface is not stable and the CTLA-4 molecule is continuously internalized in a clathrin dependent way, degraded in lysosomes and recycled to the cell surface[19]. CTLA-4 binds CD80 and CD86 with a 10-20 fold higher affinity compared to CD28[20] and consequently outcompetes CD28 mediated activation[21]. Furthermore, CTLA-4 has an advantage in engaging to B7 molecules as it binds divalently, while CD28 binds monovalently[22]. In contrast to CD28 signalling, the CTLA-4 pathway has a suppressive character, and CTLA-4 deficient mice develop severe lymphoproliferative disease and die 3 to 4 wk after birth[23]. Of note, CTLA-4 KO mice deficient in B7-1 and B7-2, as well as CTLA-4 KO mice with a defective CD28 receptor are protected from this fatal disease[24,25]. This suggests that CTLA-4 selectively regulates CD28 mediated activation. Binding of CTLA-4 to its ligands recruits phosphatases (SHP-1, SHP-2 and PP2A), which inhibit TCR phosphorylation and several other pathways such as the PKB/Akt activation as well as the phosphorylation of extracellular-signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK)[26]. This reduces the production of IL-2 and its receptor, inhibits T cell proliferation, and consequently results in termination of the immune response[18,27].

Other members of the B7 and CD28 superfamilies: Other members of the B7 superfamily, which have been studied extensively, are the inducible costimulator ligand (ICOSL, CD275, B7h or B7-PR-1), which binds to ICOS (CD278) and the programmed death ligands 1 and 2 (PD-L1 and PD-L2) which binds to programmed death 1 (PD 1). ICOS is structurally and genetically related to CD28 and up-regulated in the course of activation[28]. ICOSL is expressed on APCs and some non-hematopoietic cells (*e.g.*, endothelial cells). Different from CD28/B7 signalling, ICOS/ICOSL interaction is not essential for T cell activation, but rather acts by fine-tuning effector T cell differentiation and cytokine production[29]. Furthermore, ICOS is crucial for germinal centre formation and class switching in B cells[30,31].

PD-1 is a suppressive member of the CD28 superfamily. Different from CD28 and CTLA-4, PD-1 is not expressed as a dimer and its expression is not limited to T cells. It can be found on activated T cells, but also on B cells and myeloid cells, which suggests a broader spectrum of regulation compared to CTLA-4[32]. Ligation to PD-L1 and PD-L2, which are expressed on activated APCs, inhibits cytokine production and leads to cell cycle arrest[33,34]. Furthermore, PD-1 signalling was found to be involved in $CD8^+$ T cell differentiation and regulation[35].

The CD40/CD40L interaction: CD40 (TNFRSF5) is a type I trans-membrane protein, which clusters upon engagement to its ligand CD40L (CD154, TNFSF5, gp39, T-BAM, or TRAP)[36]. CD40 ligation further induces the recruitment of adaptor proteins (TNF-associated factors), which then in turn trigger several possible pathways including the canonical and non-canonical nuclear factor κB (NFκB) signalling pathway, the mitogen activated protein kinases (MAPK), the phosphoinositide 3-kinase (PI3K) and the phospholipase Cγ (PLCγ) pathways[37]. CD40 is constitutively expressed on APC and on many other cell types including non-hematopoietic cells (*e.g.*, fibroblasts and epithelial cells)[38]. CD40L forms a sandwich structure composed of a β-sheet, an α-helix loop and another β-sheet and is expressed as a trimeric complex on activated T cells and platelets[39]. Under inflammatory conditions it can also be found on natural killer (NK) cells, mastocytes and eosinophils[38]. Its expression on T cells is mainly restricted to $CD4^+$ T helper (Th) cells, but there is also a small population of $CD8^+$ T cells and γδ T cells which can express CD40L[36]. Furthermore, it has been shown that CD40L is expressed on $CD8^+$ T cells in the presence of IL-12 and that these cells potentially represent a $CD8^+$ T helper cell subset[40,41].

Upon activation, CD40L is up-regulated as early as 5 to 15 min after stimulation and reaches a maximum after 6 to 8 h[36]. This fast up-regulation is made possible *via* preformed CD40L (pCD40L), which is stored in lysosomal compartments and can be mobilised in response to an activation signal[42].

The broad expression of CD40 suggests involvement in many different immune modulatory mechanisms. In this context, CD40L engagement to CD40 results in increased survival of APC[43], production of cytokines[44], up-regulation of B7 molecules and nitric oxide (NO) production[45] and is critical for full maturation of dendritic cells (DC)[46]. Furthermore, CD40 signalling is crucial for B cell activation and differentiation, antibody production, immunoglobulin-class switching and germinal centre formation[47,48]. CD40/CD40L KO mice do not only show hyper-IgM syndrome, but also exhibit deficiency in priming of T cells[36]. Signalling *via* CD40/CD40L results in enforcement of the CD28-B7 interaction and antigen presentation and is crucial for expansion and maturation of effector T (Teff) cells[38,49]. Furthermore, CD40/CD40L mediated contact between $CD4^+$ T helper cells and professional APC (DC) is important to enable DC to subsequently prime $CD8^+$ cytotoxic T lymphocytes (CTL)[50].

Other members of the TNF and TNFR superfamilies: Other members of the TNF/TNFR superfamily have gained importance during the last years. Among those are the interactions between the glucocorticoid-induced tumour necrosis factor related receptor (GITR) and its ligand GITR-L, between OX40 (CD134 or TNFRSF4) and OX40 ligand (OX40L, CD252 or TNFSF4), between 4-1BB (CD137 or TNFRSF9) and 4-1BB ligand (4-1BBL or TNFSF9) and CD27 (TNFRSF7) and CD70 (TNFSF7). In general, these

TNF/TNFR superfamily members are up-regulated or induced upon activation on T cells and their ligands on APCs. Signalling *via* these pathways regulates the frequency of effector or memory cells, provides proliferation and survival signals and promotes cytokine production[51]. The expression of OX40L, 4-1BBL and CD70 on non-immune cells (*e.g.*, endothelial cells or smooth muscle cells) further suggests a role in tissue inflammation in different disease settings[52,53]. In addition, TNF/TNFR superfamily members are expressed on natural killer (NK) and natural killer T (NKT) cells and signalling increases their effector function[51].

Regulatory T cells

A subset of $CD4^+$ T cells has regulatory capacity. In a healthy individual they constitute about 10% of circulating $CD4^+$ T cells. Treg cells play a key role in dampening of immune responses, prevention of autoimmune and allergic diseases, as well as in tolerance after transplantation[54]. They are characterized by constitutive expression of the IL-2 receptor α-chain CD25, CTLA-4 and the forkhead transcription factor Foxp3[55,56]. The latter one is crucial for the suppressive function of Treg cells, as ectopic expression of Foxp3 can induce regulatory function in naïve T cells[57]. Loss of Foxp3 results in impairment of Treg cells and in autoimmune disorders in mice (Scurfy)[58] and humans (IPEX-syndrome)[59].

Two subgroups of Foxp3 expressing Treg cells have been identified: the so called thymus derived Treg cells (tTreg) and induced Treg cells (iTreg), which are generated in the periphery from naïve $CD4^+$ T cells. *In vitro,* iTreg cells can be induced by antigenic stimulation in the presence of IL-2 and TGF-β[60,61]. Although the situation *in vivo* is less clear, iTreg cells are thought to be generated under non-inflammatory conditions in the presence of IL-2 and TGF-β by chronic sub-optimal antigen exposure[62-64], *e.g.*, by recognition of an antigen on immature DC which do not provide costimulation[65]. Furthermore, a role for retinoic acid (RA), which increases TGF-β production and favors Foxp3 polarization, has been unraveled[66,67]. During an acute inflammation (*e.g.*, in allergic or autoimmune diseases or during the course of an infection), in the presence of high amounts of inflammatory cytokines, the generation of Teff cells is favored over Treg cell induction[68].

Unfortunately it is not yet possible to distinguish tTreg and iTreg cells since both of them express CTLA-4, CD25 and Foxp3. Helios (a member of the Ikaros transcription factor family) and Neuropilin-1 (Nrp 1) have been suggested as specific markers for tTreg cells, but controversial findings regarding their expression on tTreg *vs* iTreg cells limit their use as reliable markers[69-72].

There are also $CD4^+$ Treg cell subtypes induced in the periphery which do not express Foxp3. Among those are T regulatory cells 1 (Tr1), which can be induced from naïve $CD4^+$ T cells in the presence of IL-10[73] and T helper cells type 3 (Th3), which require TGF-β[74]. Up to now, it is difficult to identify those Treg cell subsets by means of a specific surface marker. Therefore, they are predominantly defined by their cytokine profile. Tr1 cells are characterized by a high IL-10 and TGF-β production, low levels of IL-2, variable levels of IL-5 and IFN-γ and no IL-4[73]. Th3 cells produce mainly TGF-β and variable levels of IL-10 and IL-4[75].

Activation and expansion of Treg cells requires a TCR signal *in vitro*[76,77] and *in vivo*[78,79] and is consequently antigen specific. Whether or not they suppress in an antigen-specific way is still a matter of debate. A key molecule in suppression by Treg cells is CTLA-4. Mice which display a Treg-specific deficiency in CTLA-4 develop severe autoimmune diseases, and Treg cells from these mice show reduced suppressive capacity *in vitro*[80]. In contrast to conventional T cells, Treg cells express CTLA-4 constitutively[81] and therefore have a natural advantage over naïve T cells in terms of CD80/CD86 engagement. In addition, CTLA-4 expressed by Treg cells also has a cell-extrinsic mechanism of action. It has been demonstrated by Qureshi and coworkers that CTLA-4 engagement to the B7 molecules leads to trans-endocytosis and degradation of CD80 and CD86 on the surface of APCs[82]. This effect can only be mediated by CTLA-4 expressed on the cell surface, but not by soluble CTLA-4. As a result, the availability of B7 receptors and consequently the CD28 mediated activation of T cells are reduced. Moreover, CTLA-4/B7 interaction might lead to "reverse signalling" in APC. In the course of CTLA-4 engagement, APC start to produce indoleamine 2,3-dioxygenase (IDO), which catalyses the degradation of tryptophan and thus creates a local inhibitory environment for T cells[83]. This also induces the nuclear translocation of the transcription factor Foxo3[84], which inhibits the production of IL-6 and of tumor necrosis factor alpha (TNFα) but increases the secretion of suppressive cytokines such as IL-10[85]. Apart from mechanisms mediated by direct cell contact to APCs, Treg cells also secrete suppressive molecules such as IL-10[86], TGFβ[87] and IL-35[88] and molecules which can directly kill Teff cells, such as granzyme B and perforin[89]. Membrane-bound TGFβ[90] or production of cyclic adenosine monophosphate (cAMP), which can be transferred to Teff cells *via* gap junctions, can suppress Teff cells *via* direct cell-cell contact[91]. Other suppressive mechanisms involve CD39 and CD72 mediated degradation of adenosine monophosphate (AMP) and adenosine triphosphate (ATP) to adenosine[92] or suppression by Galectin-1[93]. Finally, Treg cells are thought to suppress Teff cells by IL-2 deprivation and subsequent apoptosis[94]. IL-2 is crucial for Treg cell generation, induction and maintenance[95], but, in contrast to Teff cells, Treg cells lack the ability to produce IL-2 and are consequently dependent on an external source[96]. Since Treg cells constitutively express the high affinity receptor for IL-2 (CD25)[55], they have an advantage over Teff cells in terms of binding IL-2. In an inflammatory setting, however, when Teff cells also up-regulate CD25, this advantage is lost. Therefore, it was suggested that suppression by IL-2 consumption is predominantly important

in steady-state conditions as a feed-back mechanism to prevent Treg cell overgrowth and not in an inflammatory setting[97].

Since none of the above described mechanisms results in a complete absence of regulatory activity when deleted, there is most likely not one core-mechanism of suppression. In this context, Treg-specific CTLA-4 deficiency resulted in systemic autoimmune diseases[80], but transfer of CTLA-4 deficient Treg cells could prevent experimental colitis *in vivo*[98] and IL-10 deficient Treg cells are able to suppress auto-immunity, but cannot prevent experimental colitis[86,99]. Thus, Treg cells can compensate for defects and adapt to environmental circumstances.

THE EFFECTS OF BLOCKING COSTIMULATORY SIGNALS

Since the "second" or "costimulatory" signal is of great importance for the activation and successful differentiation of naive T cells into fully functional Teff cells[6], blocking these pathways presents a promising approach to treat T cell mediated autoimmune diseases (*e.g.*, rheumatoid arthritis or multiple sclerosis), transplant rejections or graft *vs* host disease (GvHD). Compared to conventional immunosuppressive drugs, costimulation blockade provides the advantage of selective inhibition of T cell responses and has the potential of inducing long-lasting antigen-specific tolerance[100]. The most promising and best studied candidates for such manipulations are the CD28/B7 and CD40/CD40L pathways as they are both critical for T cell activation.

Blocking the CD28/B7 pathway using CTLA-4Ig

Up to now, the most promising candidate to achieve CD28/B7 costimulation blockade is the CTLA-4Ig fusion protein. It consists of the extracellular domain of the CTLA-4 molecule fused to the Fc-region of IgG. CTLA-4-Ig binds both B7 molecules with the same high binding affinity as CTLA-4. The effect of CTLA-4Ig has first been demonstrated in an animal model of islet transplantation, where CTLA-4Ig treatment led to long-term acceptance of xenografts[101]. Also in systems of allogeneic islet or cardiac transplantation or graft *vs* host disease (GvHD), CTLA-4Ig could prolong survival and reduce rejection[102-104]. Furthermore, CTLA-4Ig is a potent immunosuppressor in animal models of autoimmunity such as experimental autoimmune encephalomyelitis (EAE)[105], diabetes[106] and systemic lupus erythematodes (SLE)[107].

CTLA-4Ig has also been used effectively in clinical trials. Davies and co-worker showed that tolerizing bone marrow cells *ex vivo* in the presence of CTLA-4Ig prior to transplantation to a MHC-matched recipient reduces the incidence of acute and chronic GvHD[108]. Furthermore, CTLA-4Ig (abatacept) treatment in combination with cyclosporin and methotrexate prevents acute GvHD after hematopoietic cell transplantation from an unrelated donor[109]. Since 2005, CTLA-4Ig (abatacept) is approved by the FDA for the treatment of rheumatoid arthritis (RA)[110] and a second-generation molecule (belatacept) with higher binding affinity for B7-1 and B7-2 was approved in 2011 to prevent rejection after renal transplantation[111].

Blocking the CD40/CD40L pathway

Antagonistic anti-CD40L monoclonal antibodies (mAb) have shown impressive effects in many animal models. Blocking CD40L prevents acute and chronic GvHD[112]. If given at the time of transplantation, anti-CD40L treatment prolongs graft survival in a model of heart, islet, liver and limb transplantation[113-116]. Targeting the CD40L receptor proved to be efficient in animal models of autoimmune diseases such as EAE, arthritis, SLE, colitis and arteriosclerosis[117]. However, clinical trials with an anti-CD40L mAb (Ruplizumab) in SLE patients have led to thromboembolic side-effects and had to be halted[118]. This effect was caused by the Fc-fragment of the antibody bound to a receptor on platelets which also express CD40L. Nonetheless, the findings in animal systems are extremely promising and, consequently, it is attempted to find alternative ways to achieve CD40L blockade. mAb with an engineered, aglycosylated or mutated Fc-part were created[119-121]. The modifications alter the antibody in a way that Fc-receptor or complement mediated platelet aggregation and subsequent thromboembolic events are prevented. Furthermore, alternative blocking reagents such as small molecules or peptides are currently explored[122,123].

The CD40/CD40L interaction can also be interrupted by targeting the CD40 receptor. A human antagonistic anti-CD40 antibody showed some effect in *ex vivo* studies[124,125] and proved to be safe in a Phase I clinical trial on lymphocytic leukaemia patients[126]. Another antagonistic anti-CD40 antibody, chimeric 5D12, was tested successfully in an EAE model in marmoset monkeys[127]. Furthermore, we showed that 5D12 was well tolerated in a phase I clinical trial in patients with Crohn's disease[128]. However, CD40 is expressed on many different cell types and consequently targeting this molecule might have broad and undesired effects. Additionally, most antibodies directed against CD40 are stimulatory for APC and B cells by cross-linking the trimeric receptor.

Combined blockade of the CD28/B7 and the CD40/CD40L pathway

Although CTLA-4Ig and anti-CD40L antibodies show great potential in various disease models, the combination of both is often superior. It is indeed possible that in the absence of CD40L or CD28 triggering, the T cell can still receive sufficient activation signals from other costimulatory pathways[129,130]. Especially in animal models of solid organ transplantation, combined blockade of CD28/B7 and CD40/CD40L is required for permanent tolerance induction in mice[131] and non-human primates[132]. Also, in animal models of leukaemia[133] or autoimmune diseases such as EAE[134] and SLE[135], the combination of CTLA-4Ig and MR1 (an anti-CD40L mAb) could more effectively reduce disease symptoms than both alone.

We made similar observations in a fully MHC mismatch model of GvHD with allogeneic bone marrow transfer. In our study, only the combined blockade of the CD28/B7 pathway (using CTLA-4Ig) and the CD40/CD40L pathway (using MR1) prevented lethal GvHD and resulted in long-lasting tolerance and the induction of stable mixed chimerism[136].

Mechanisms of suppression by CD28/CTLA-4/B7 and CD40/CD40L blockade

The mechanisms of tolerance induction by costimulation blockade, in particular of the CD28/CTLA-4/B7 and the CD40/CD40L interaction, have extensively been studied in allo-responses such as GvHD or transplant rejection. In these settings, deprivation of necessary activation signals (CD28 and/or CD40 triggering) leads to T cell hyporesponsiveness[8], which is followed by peripheral clonal deletion[136,137]. Elimination of the hypo-responsive T cells is predominantly mediated by apoptosis[138-140]. In a fully miss-matched transplantation model, the tolerising effect of combined CD28/B7 (using CTLA-4Ig) and CD40/CD40L (using MR1) blockade can be reversed by the calcineurin inhibitor cyclosporine A (CsA), which prevents apoptosis[138]. In contrast, rapamycin (which favours apoptosis) acts synergistically with costimulation blockade. While activation induced cell death (AICD) seems not to be essential, passive cells death is crucial for the induction of tolerance under the cover of CTLA-4Ig and MR1. Heart allografts were rejected in Bcl-xL deficient mice despite costimulation blockade[139], but Fas-deficiency was not able to break tolerance[140]. Additionally, CTLA-4Ig has been suggested to act *via* reverse signalling to APCs and to induce IDO production, which contributes to creating a suppressive environment[141].

THE ROLE OF TREG CELLS IN IMMUNE SUPPRESSION BY COSTIMULATION BLOCKADE

Although apoptosis of Teff cells after activation in the absence of costimulatory has been demonstrated by many research groups, complete deletion of responsive T cells takes several weeks[137] while tolerance can already be observed shortly after treatment[142]. In this context, it has been demonstrated by the group of Waldmann that $CD4^+$ cells, which have been tolerized to allo-antigens by CD40L blockade, are not only hypo-responsive but moreover display a suppressive function[143,144]. Therefore, it has been suggested that Treg cells, at least partially, mediate tolerance until Teff cells have been eliminated. In line with this, it has been demonstrated that tolerance induction by CD40L or B7 blockade is abrogated when Treg cells are depleted. In a study performed by Taylor and co-workers, $CD4^+$ cells were tolerized to allo-antigens *ex vivo* in the presence of antagonistic anti-CD40L or anti-B7 antibodies. Transfer of these cells to animals suffering from GvHD did abrogate the disease. However, if Treg cells were depleted prior to the transfer, GvHD was not suppressed[145]. Also, long-term acceptance of a skin or a heart allograft under the cover of CD40L blockade could be abrogated if recipient Treg cells were depleted[146,147]. However, Kurtz *et al*[148] showed that it is possible to induce mixed chimerism after allogeneic bone marrow transplantation under the cover of CD40L blockade, but they did not find evidence for an involvement of Treg in this system. In line with this, we have previously shown in a model of GvHD with allogeneic bone marrow transplantation that tolerance induction by combined CD40/CD40L and CD28/B7 blockade and the development of mixed chimerism are still possible despite the absence of donor Treg cells[136]. In both studies T cell hypo-responsiveness and deletion were the main mechanisms by which tolerance was achieved. The importance of Treg cells for tolerance induction by costimulation blockade thus might depend on the disease model. The recipient Treg cells might be important in the setting of a solid organ transplant, while in GvHD the presence of Treg cells within the donor cell transplant might not be crucial for the outcome of the disease.

Costimulatory requirements of Treg cells

Involvement of Treg cells in tolerance induction by costimulation blockade implies that Teff cells and Treg cells have different requirements regarding costimulation. Such different requirements could result in differential modulation of Teff cells and Treg cells by costimulation blockade. Both cell types share the TCR-mediated recognition of an antigen as the first signal for activation. However, the costimulatory requirements for Treg cells are less clear than those for Teff cells (Figure 1). CD28/B7 signalling is crucial for thymic Treg cell generation and homeostasis since mice deficient in CD28 or B7 molecules have a significantly reduced number of Treg cells in the thymus as well as in the periphery[149,150]. CD40L and glucocorticoid-induced tumour necrosis factor related receptor (GITR) signalling also play an important role during thymic development of Treg cells[151-153]. Whether CD28 and/or CD40L costimulation is equally important for the activation or the induction of Treg cells in peripheral lymphoid organs as it is for Teff cells, however, is still a matter of debate. We have shown that blocking the B7 molecules using anti-B7-1 and anti-B7-2 antibodies in combination with an antagonistic anti-CD40 antibody resulted in human T cell hypo-responsiveness *in vitro*. This effect was associated with the induction of a T cell subset with suppressive activity, which expressed high levels of ICOS and produced IL-10[154]. Furthermore, we have shown that the beneficial effect of combined CTLA-4Ig and MR1 treatment in a mouse model of GvHD is associated with an increase in the frequency of $Foxp3^+$ Treg cells between day 6 and 30 after T cell transfer[136]. Both findings argue for costimulation independent Treg induction and expansion. We further conducted a more detailed examination of the effect of CTLA-4Ig and MR1 on murine Treg cells *in vitro*. Here, we showed that Treg cells can proliferate

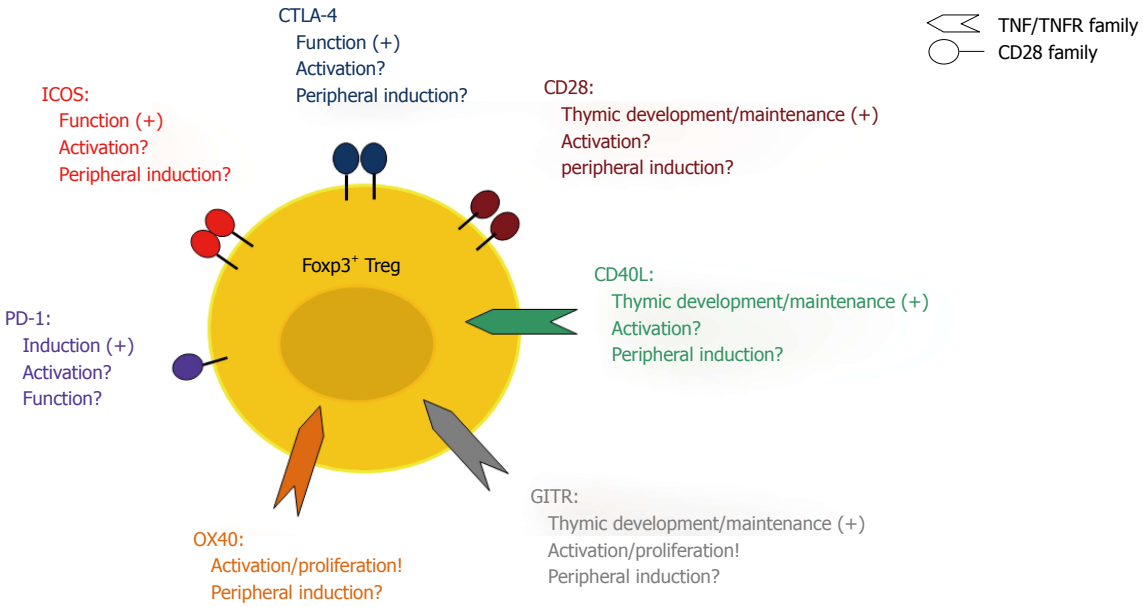


Figure 1 Costimulatory requirements of Foxp3$^+$ Treg cells. Treg cells, similar to Teff cells, depend on T cell receptor (TCR)-mediated recognition of an antigen for activation (signal 1). The requirements of Treg cells regarding the second, costimulatory signal are less clear. The exact pathways and necessary signals are still a matter of debate. So far, it is well established that Treg cells depend on CD28 and CD40L for their thymic development. Also, glucocorticoid-induced tumour necrosis factor related receptor (GITR) is stabilizing Foxp3 expression during maturation in the thymus. In order to get properly activated and to proliferate, triggering of OX40 and GITR, in concert with IL-2, was reported to be crucial. The induction of iTreg cells in the periphery is promoted by PD-1 signalling. The function of Treg cells depends on cytotoxic T lymphocyte antigen (CTLA)-4 and ICOS. See text for more details and references.

and be activated if CTLA-4Ig and MR1 were added to the cultures at a dose where Teff cells are inhibited[155]. Also other laboratories, in which the blockade of the CD28/B7 and/or the CD40/CD40L interaction was studied, have observed an increase of functional Treg cells *in vitro*[145,156]. Furthermore, a selective non-cross-linking CD28 antagonist induced tolerance to renal and cardiac allografts in non-human primates and this was associated with an increased frequency of Foxp3$^+$ Treg cells[157]. In a mouse model of heart transplantation under the cover of an anti-CD40L mAb, Treg cell were functional and crucial to prevent rejection[147]. Altogether, these findings suggests that Treg cells are less dependent on CD28/B7 and CD40/CD40L costimulation compared to Teff cells and can therefore still be activated and expand in the presence of CTLA-4Ig and MR1.

However, Treg cells are probably not completely independent of CD28/B7 and CD40/CD40L costimulation. In this context, we showed that the increase in the Treg cell frequency *in vitro* observed in the presence of CTLA-4Ig and MR1 is dependent on the concentration of the blocking agents. While a low dose of CTLA-4Ig and MR1, ranging between 0.125 μg/mL and 4 μg/mL, resulted in a concentration dependent increase in the frequency of Treg cells, a higher dose (between 8 μg/mL and 32 μg/mL) resulted in a concentration dependent decrease in the frequency of the Treg cells (manuscript in preparation). Thus, at a very high dose of costimulatory blocking agents, Treg cells also seem to be affected. We further explored this issue in a mouse model of GvHD. A treatment regime using 500 μg (per mouse) of CTLA-4Ig (in combination with MR1) was equally effective as a 10 times lower dose in preventing the disease. However, intermediate doses had no effect on survival. Again, the treatment with a low dose of CTLA-4Ig, but not with a high dose, was followed by an increase in Treg cell frequency (manuscript in preparation). This observation can potentially be explained assuming two separate mechanisms of action (Figure 2): treatment with a high dose blocks all the Teff cells (but also the Treg cells) and therefore prevents the disease. At a low dose, however, not all the Teff cells are blocked, but Treg cells remain activated and are able to suppress the remaining Teff cells. Intermediate doses are not effective, most likely because not all the Teff cells are blocked while at the same time Treg cells are affected and therefore not able to suppress Teff cells. It is possible that Treg cells need the same costimulatory signals as Teff cells, but have a lower threshold for activation. Another possibility is that a low dose of CTLA-4Ig and MR1 only partially blocks the Teff cells, which produce low amounts of IL-2. As Treg cells can take up IL-2 more efficiently than Teff cells due to the constitutive expression of the high affinity IL-2 receptor (CD25)[95], the low amounts of IL-2 might be sufficient to maintain Treg cells but not enough to allow for Teff cell priming and activation. This issue will have to be examined more closely in the future. If IL-2 and not costimulation is the limiting factor for Treg cell activation, expansion of Treg cells can be facilitated by adding exogenous IL-2.

Other costimulatory pathways have been suggested to be relevant for Treg cell activation and function. Triggering GITR on Treg cells increases their proliferation and enforces their suppressive activity[158]. Blocking the

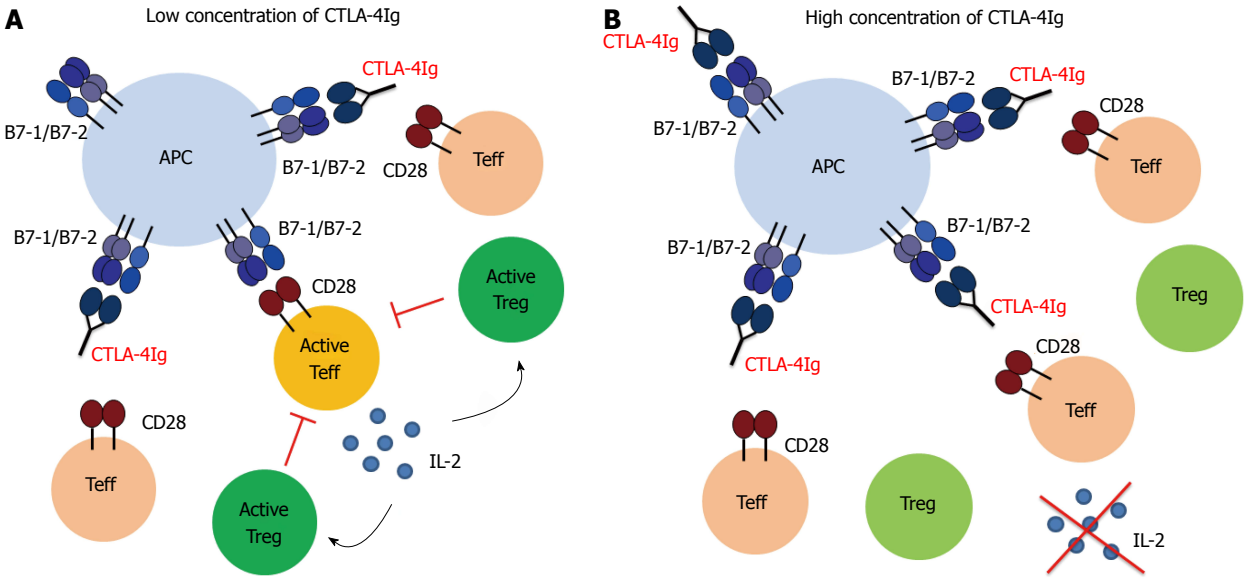


Figure 2 The potential effects of a cytotoxic T lymphocyte-associated antigen-4Ig treatment started before T cell priming. If cytotoxic T lymphocyte antigen (CTLA)-4Ig is given before T cell priming at a low dose, it reduces CD28 mediated T cell activation. Since blockade of B7 molecules is not complete, some Teff cells are still activated and produce interleukin (IL)-2. Treg cell-activation is less or not dependent on CD28 signalling and the low amounts of IL-2 produced by the Teff cells are sufficient to maintain Treg cell activation. In that way activated Treg cells further suppress the remaining Teff cells. The effect of CTLA-4Ig is in this situation based on down-regulation of Teff cells and maintenance of Treg activity (A). If CTLA-4Ig is given at a high concentration, Teff and Treg cells are equally suppressed due to missing costimulation and/or IL-2. The effect of CTLA-4Ig is in this case based on reduced Teff cell activity (B). APC: Antigen presenting cell, Teff: effector T cell, Treg: Regulatory T cell.

ICOS/ICOSL interactions in a model of ovalbumin (OVA) induced airway inflammation[159] and EAE[30] abrogated Treg activity *in vitro* and *in vivo*. An antagonistic anti-PD-1 antibody can prevent the induction of Treg cells from naive $CD4^+$ T cell *in vitro*, which suggests that PD-1 signalling is important in this process[160]. Defects in or blockade of CTLA-4 leads to uncontrolled expansion of Treg cells, which suggests a cell-intrinsic effect of CTLA-4 triggering on Treg cells and an important role for CTLA-4 in regulating Treg generation in the thymus and in the periphery[161,162]. Also, CTLA-4 regulates the TCR specificity during thymic development as over-expression of CTLA-4 leads to a self-skewed TCR repertoire whereas deficiency of CTLA-4 prevents the development of a self-skewed TCR repertoire[163]. There is also evidence that CTLA-4 signalling is involved in the induction of Foxp3 in naïve T cells and promotes generation of iTreg cells in the periphery[164]. In addition, CTLA-4 is a key mediator in suppression by Treg cells as described before. Recently, the OX40/OX40L pathway has come into focus with regard to Treg cell activation and proliferation. OX40 triggering acts in concert with IL-2 and leads to extensive Treg cell expansion. In the presence of IL-2, these cells are stable and show potent suppressive activity[165].

The effect of CD40/CD40L blockade on Treg cells

A large body of evidence including our own studies suggests that Treg cells are not affected by CD40/CD40L blockade[120,136,143-147,155]. Although Treg cells require CD40L signalling during their development in the thymus[152,153], only about 4%-9% of Treg cells express CD40L in the periphery[166]. Up-regulation of CD40L in Treg cells upon activation is delayed compared to Teff cells, which express CD40L within the first 5 to 15 min after activation[36]. This fast up-regulation is made possible through the storage of preformed CD40L (pCD40L). Treg cells, on the other hand, are incapable of storing pCD40L and consequently have to generate it *de novo*[42,166]. Altogether this suggests that Treg cells are indeed not dependent on CD40L signalling concerning their activation. Therefore, CD40L blockade provides a promising target to modulate the balance between Treg cells and Teff cells in favour of Treg cell activity.

The effect of CTLA-4Ig on Treg cells

CTLA-4Ig has been proven to be very effective as an immunosuppressive treatment in various animal models and is successfully used in the clinic to treat rheumatoid arthritis (abatacept) and rejection after renal transplantation (belatacept)[110,111]. However, recent findings have raised concern about the use of CTLA-4Ig in systems where Treg cells are crucial for the success of the therapy. Riella and co-workers showed that CTLA-4Ig accelerates transplant rejection in a MHC class Ⅱ mismatch model, in which tolerance induction and graft survival is crucially dependent on Treg cell function[167]. Furthermore, in a study in which rejection of a skin transplant could be prevented by expansion of Treg cells using IL-2/anti-IL-2 complexes, simultaneous administration of CTLA-4Ig could break tolerance induction[168]. As mentioned before, we have observed a dose dependent effect of CTLA-4Ig on Treg cells (manuscript in preparation). It is possible that the amount of CTLA-4Ig applied was indeed high

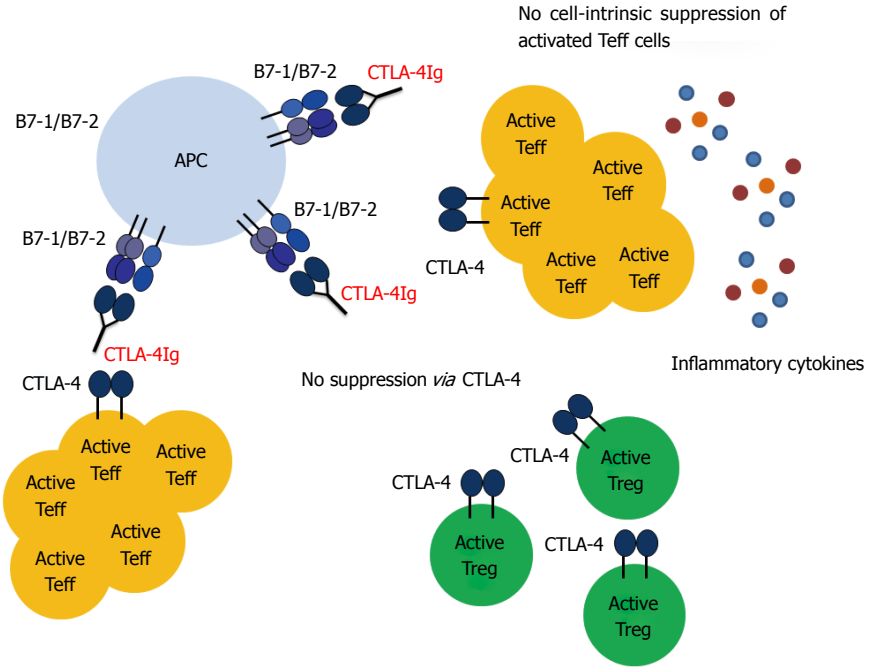


Figure 3 The potential effects of cytolytic T lymphocyte-associated antigen-4Ig treatment given after T cell priming. If cytolytic T lymphocyte antigen (CTLA)-4Ig is applied after priming when T cells are already activated, it mainly interferes with the CTLA-4/B7 interaction. This interaction is important for cell-intrinsic down-regulation of Teff cells. In the absence of this cell-intrinsic regulation, activated Teff cells proliferate more strongly and secrete more inflammatory cytokines. Furthermore, Treg cells are not able to exert their suppressive function mediated *via* CTLA-4. Both effects will potentially result in further increase of the T cell mediated inflammation. APC: Antigen presenting cell; Teff: Effector T cell; Treg: Regulatory T cell.

enough to interfere with the Treg cells. Especially in a model where Treg cells are crucial for the outcome of the disease, a high dose might be less effective than a low dose which spares the Treg cells.

The differential sensitivity of Treg cells *vs* Teff cells to CD28/CTLA-4/B7 blockade is certainly not the only problem that might arise from CTLA-4Ig treatment. Another factor that has to be considered is that CTLA-4Ig does not only interfere with the CD28/B7 signaling but also with the CTLA-4/B7 signaling (Figure 3). CTLA-4 is expressed on activated Teff cells and constitutively on Treg cells, and triggering of membrane CTLA-4 leads to suppression of the corresponding T cell[18,20]. This holds true for Teff cells as well as for Treg cells[169]. Since Treg cells express CTLA-4 constitutively, CTLA-4Ig administration during priming will presumably prevent CTLA-4 mediated cell-intrinsic suppression of Treg cells and will therefore enhance their activity. In addition, CTLA-4Ig engagement to the B7 ligands leads to reverse signalling to the APCs, which results in IDO production[141]. Both mechanisms thus result in the creation of a suppressive environment. However, CTLA-4 is a also key molecule for Treg cell function[81]. Our above mentioned data argue against interference of CTLA-4Ig with Treg cell activation, but do not exclude interference with Treg function or induction. In this context, blockade of the B7 molecules with CTLA-4Ig prevents CTLA-4 mediated trans-endocytosis and degradation of the B7 molecules by Treg cells as well as "reverse signalling" *via* CTLA-4/B7 signalling and IDO production. Moreover, if CTLA-4Ig is given after T cell priming, Teff cells will also have up-regulated CTLA-4 and by blocking B7 molecules, the cell-intrinsic suppression of Teff cells might be blocked. This is not relevant in a setting of transplantation, when it is exactly known when T cell priming occurs. However, for patients with autoimmune diseases such as multiple sclerosis (MS), the situation is different. It is not possible to predict disease onset or a relapse episode and therefore it is not known when auto-reactive T cells are primed and activated. In such settings it might be dangerous to apply CTLA-4Ig treatment. Indeed, we have found in a model of experimental autoimmune encephalomyelitis (EAE), the mouse model for the human disease MS, that treatment with CTLA-4Ig after T cell priming leads to exacerbation of the disease. This is most likely due to interference with the CTLA-4/B7 mediated suppression (manuscript in preparation). Further studies will be required to examine if this exacerbation is a result of missing cell-intrinsic suppression of the Teff cells, interference with Treg cell function and *de novo* induction or both.

CONCLUSION

Based on the above discussed studies and our own results we believe that it can be possible to modulate costimulation in such a way that Teff cell activation is prevented but Treg cells can still be activated. Especially blockade of the CD40/CD40L pathway provides a promising target to manipulate the Teff/Treg cell balance in favor of Treg cell activity. However, blockade of the CD40/CD40L interaction alone is not always sufficient to guar-

antee full protection. Therefore, CD40/CD40L blockade must be combined with CTLA-4Ig in order to prevent CD28 mediated activation. Several factors have to be taken into account when using CTLA-4Ig as a treatment option. First, if CTLA-4Ig is given before T cell priming (*e.g.*, in a transplant setting), the dose of the reagent is an important factor. A high dose of CTLA-4Ig can also affect the Treg cells. Careful titration is required to find the optimal dose that blocks Teff cells but spares the Treg cells (Figure 2). This might be of great importance if Treg cells are crucial for the success of the therapy. Second, it has to be considered whether CTLA-4Ig is given before or after T cell priming. CTLA-4Ig treatment after T cell priming might be dangerous as it can interfere with CTLA-4 mediated suppression (Figure 3). This can affect cell-intrinsic suppression of the Teff cells and/or affect Treg cell function and induction. Third, knowing the pathophysiology of the disease (especially concerning involvement of Treg cells) is crucial in order to find a balance between maximal suppression of Teff cells and minimal interference with Treg cells.

It will be important to more closely study the costimulatory requirements of Treg cells and the effect of blocking those signals on their activity. This will help to improve the success of a therapy involving costimulation blockade. Especially when using CTLA-4Ig, it will be necessary to know exactly which effect the treatment has in the corresponding disease setting in order to prevent undesired effects. Furthermore, the finding that Treg cells and Teff cells respond differently to costimulation blockade can potentially be exploited in a context of Treg cells based therapy. Treg cells can be expanded *in vitro* or perhaps even *in vivo*, while the outgrowth of Teff cells is prevented under the cover of costimulation blockade.

REFERENCES

1 **Starr TK**, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annu Rev Immunol* 2003; **21**: 139-176 [PMID: 12414722 DOI: 10.1146/annurev.immunol.21.120601.141107]

2 **Hogquist KA**, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* 2005; **5**: 772-782 [PMID: 16200080 DOI: 10.1038/nri1707]

3 **Schwartz RH**. T cell anergy. *Annu Rev Immunol* 2003; **21**: 305-334 [PMID: 12471050 DOI: 10.1146/annurev.immunol.21.120601.141110]

4 **Grillot DA**, Merino R, Núñez G. Bcl-XL displays restricted distribution during T cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. *J Exp Med* 1995; **182**: 1973-1983 [PMID: 7500043]

5 **Lenardo MJ**. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature* 1991; **353**: 858-861 [PMID: 1944559 DOI: 10.1038/353858a0]

6 **Bretscher PA**. A two-step, two-signal model for the primary activation of precursor helper T cells. *Proc Natl Acad Sci USA* 1999; **96**: 185-190 [PMID: 9874793]

7 **Liu Y**, Linsley PS. Costimulation of T-cell growth. *Curr Opin Immunol* 1992; **4**: 265-270 [PMID: 1418704]

8 **Mueller DL**, Jenkins MK, Schwartz RH. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol* 1989; **7**: 445-480 [PMID: 2653373 DOI: 10.1146/annurev.iy.07.040189.002305]

9 **Sharpe AH**, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol* 2002; **2**: 116-126 [DOI: 10.1038/nri727]

10 **Lenschow DJ**, Su GH, Zuckerman LA, Nabavi N, Jellis CL, Gray GS, Miller J, Bluestone JA. Expression and functional significance of an additional ligand for CTLA-4. *Proc Natl Acad Sci USA* 1993; **90**: 11054-11058 [PMID: 7504292]

11 **Hathcock KS**, Laszlo G, Pucillo C, Linsley P, Hodes RJ. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *J Exp Med* 1994; **180**: 631-640 [PMID: 7519245]

12 **McAdam AJ**, Schweitzer AN, Sharpe AH. The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. *Immunol Rev* 1998; **165**: 231-247 [PMID: 9850864]

13 **Jenkins MK**, Taylor PS, Norton SD, Urdahl KB. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J Immunol* 1991; **147**: 2461-2466 [PMID: 1717561]

14 **Appleman LJ**, Berezovskaya A, Grass I, Boussiotis VA. CD28 costimulation mediates T cell expansion via IL-2-independent and IL-2-dependent regulation of cell cycle progression. *J Immunol* 2000; **164**: 144-151 [PMID: 10605005]

15 **Viola A**, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. *Science* 1996; **273**: 104-106 [PMID: 8658175]

16 **Boise LH**, Minn AJ, Noel PJ, June CH, Accavitti MA, Lindsten T, Thompson CB. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* 1995; **3**: 87-98 [PMID: 7621080]

17 **Verbinnen B**, Van Gool SW, Ceuppens JL. Blocking costimulatory pathways: prospects for inducing transplantation tolerance. *Immunotherapy* 2010; **2**: 497-509 [PMID: 20636004 DOI: 10.2217/imt.10.31]

18 **Walunas TL**, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, Thompson CB, Bluestone JA. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1994; **1**: 405-413 [PMID: 7882171]

19 **Qureshi OS**, Kaur S, Hou TZ, Jeffery LE, Poulter NS, Briggs Z, Kenefeck R, Willox AK, Royle SJ, Rappoport JZ, Sansom DM. Constitutive clathrin-mediated endocytosis of CTLA-4 persists during T cell activation. *J Biol Chem* 2012; **287**: 9429-9440 [PMID: 22262842 DOI: 10.1074/jbc.M111.304329]

20 **Thompson CB**, Allison JP. The emerging role of CTLA-4 as an immune attenuator. *Immunity* 1997; **7**: 445-450 [PMID: 9354465]

21 **Carreno BM**, Bennett F, Chau TA, Ling V, Luxenberg D, Jussif J, Baroja ML, Madrenas J. CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression. *J Immunol* 2000; **165**: 1352-1356 [PMID: 10903737]

22 **Collins AV**, Brodie DW, Gilbert RJ, Iaboni A, Manso-Sancho R, Walse B, Stuart DI, van der Merwe PA, Davis SJ. The interaction properties of costimulatory molecules revisited. *Immunity* 2002; **17**: 201-210 [PMID: 12196291]

23 **Waterhouse P**, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, Thompson CB, Griesser H, Mak TW. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 1995; **270**: 985-988 [PMID: 7481803]

24 **Mandelbrot DA**, McAdam AJ, Sharpe AH. B7-1 or B7-2 is required to produce the lymphoproliferative phenotype in mice lacking cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). *J Exp Med* 1999; **189**: 435-440 [PMID: 9892625]

25 **Tai X**, Van Laethem F, Sharpe AH, Singer A. Induction of autoimmune disease in CTLA-4-/- mice depends on a specific CD28 motif that is required for in vivo costimulation. *Proc Natl Acad Sci USA* 2007; **104**: 13756-13761 [PMID: 17702861 DOI: 10.1073/pnas.0706509104]

26 **Bour-Jordan H**, Esensten JH, Martinez-Llordella M, Penaranda C, Stumpf M, Bluestone JA. Intrinsic and extrinsic control

of peripheral T-cell tolerance by costimulatory molecules of the CD28/B7 family. *Immunol Rev* 2011; **241**: 180-205 [PMID: 21488898 DOI: 10.1111/j.1600-065X.2011.01011.x]
27 **Krummel MF**, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 1995; **182**: 459-465 [PMID: 7543139]
28 **Hutloff A**, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczek RA. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 1999; **397**: 263-266 [PMID: 9930702 DOI: 10.1038/16717]
29 **Nurieva RI**. Regulation of immune and autoimmune responses by ICOS-B7h interaction. *Clin Immunol* 2005; **115**: 19-25 [PMID: 15870016 DOI: 10.1016/j.clim.2005.02.010]
30 **Dong C**, Temann UA, Flavell RA. Cutting edge: critical role of inducible costimulator in germinal center reactions. *J Immunol* 2001; **166**: 3659-3662 [PMID: 11238604]
31 **McAdam AJ**, Greenwald RJ, Levin MA, Chernova T, Malenkovich N, Ling V, Freeman GJ, Sharpe AH. ICOS is critical for CD40-mediated antibody class switching. *Nature* 2001; **409**: 102-105 [PMID: 11343122 DOI: 10.1038/35051107]
32 **Agata Y**, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* 1996; **8**: 765-772 [PMID: 8671665]
33 **Freeman GJ**, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000; **192**: 1027-1034 [PMID: 11015443]
34 **Latchman Y**, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, Greenfield EA, Bourque K, Boussiotis VA, Carter LL, Carreno BM, Malenkovich N, Nishimura H, Okazaki T, Honjo T, Sharpe AH, Freeman GJ. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001; **2**: 261-268 [PMID: 11224527 DOI: 10.1038/85330]
35 **Nishimura H**, Honjo T, Minato N. Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice. *J Exp Med* 2000; **191**: 891-898 [PMID: 10704469]
36 **van Kooten C**, Banchereau J. CD40-CD40 ligand. *J Leukoc Biol* 2000; **67**: 2-17 [PMID: 10647992]
37 **Bishop GA**, Moore CR, Xie P, Stunz LL, Kraus ZJ. TRAF proteins in CD40 signaling. *Adv Exp Med Biol* 2007; **597**: 131-151 [PMID: 17633023 DOI: 10.1007/978-0-387-70630-6_11]
38 **Mackey MF**, Barth RJ, Noelle RJ. The role of CD40/CD154 interactions in the priming, differentiation, and effector function of helper and cytotoxic T cells. *J Leukoc Biol* 1998; **63**: 418-428 [PMID: 9544571]
39 **Karpusas M**, Hsu YM, Wang JH, Thompson J, Lederman S, Chess L, Thomas D. 2 A crystal structure of an extracellular fragment of human CD40 ligand. *Structure* 1995; **3**: 1031-1039 [PMID: 8589998]
40 **Stark R**, Hartung A, Zehn D, Frentsch M, Thiel A. IL-12-mediated STAT4 signaling and TCR signal strength cooperate in the induction of CD40L in human and mouse CD8+ T cells. *Eur J Immunol* 2013; **43**: 1511-1517 [PMID: 23765345 DOI: 10.1002/eji.201243218]
41 **Frentsch M**, Stark R, Matzmohr N, Meier S, Durlanik S, Schulz AR, Stervbo U, Jürchott K, Gebhardt F, Heine G, Reuter MA, Betts MR, Busch D, Thiel A. CD40L expression permits CD8+ T cells to execute immunologic helper functions. *Blood* 2013; **122**: 405-412 [PMID: 23719298 DOI: 10.1182/blood-2013-02-483586]
42 **Koguchi Y**, Buenafe AC, Thauland TJ, Gardell JL, Bivins-Smith ER, Jacoby DB, Slifka MK, Parker DC. Preformed CD40L is stored in Th1, Th2, Th17, and T follicular helper cells as well as CD4+ 8- thymocytes and invariant NKT cells but not in Treg cells. *PLoS One* 2012; **7**: e31296 [PMID: 22363608 DOI: 10.1371/journal.pone.0031296]
43 **Banchereau J**, Bazan F, Blanchard D, Brière F, Galizzi JP, van Kooten C, Liu YJ, Rousset F, Saeland S. The CD40 antigen and its ligand. *Annu Rev Immunol* 1994; **12**: 881-922 [PMID: 7516669 DOI: 10.1146/annurev.iy.12.040194.004313]
44 **Foy TM**, Aruffo A, Bajorath J, Buhlmann JE, Noelle RJ. Immune regulation by CD40 and its ligand GP39. *Annu Rev Immunol* 1996; **14**: 591-617 [PMID: 8717526 DOI: 10.1146/annurev.immunol.14.1.591]
45 **Van Kooten C**, Banchereau J. CD40-CD40 ligand: a multifunctional receptor-ligand pair. *Adv Immunol* 1996; **61**: 1-77 [PMID: 8834494]
46 **Banchereau J**, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**: 245-252 [PMID: 9521319 DOI: 10.1038/32588]
47 **Rousset F**, Garcia E, Banchereau J. Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J Exp Med* 1991; **173**: 705-710 [PMID: 1705282]
48 **Kawabe T**, Naka T, Yoshida K, Tanaka T, Fujiwara H, Suematsu S, Yoshida N, Kishimoto T, Kikutani H. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity* 1994; **1**: 167-178 [PMID: 7534202]
49 **Grewal IS**, Flavell RA. The role of CD40 ligand in costimulation and T-cell activation. *Immunol Rev* 1996; **153**: 85-106 [PMID: 9010720]
50 **Bennett SR**, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 1998; **393**: 478-480 [PMID: 9624004 DOI: 10.1038/30996]
51 **Croft M**. The role of TNF superfamily members in T-cell function and diseases. *Nat Rev Immunol* 2009; **9**: 271-285 [PMID: 19319144 DOI: 10.1038/nri2526]
52 **Seko Y**, Ishiyama S, Nishikawa T, Kasajima T, Hiroe M, Suzuki S, Ishiwata S, Kawai S, Tanaka Y, Azuma M, Kobata T, Yagita H, Okumura K, Nagai R. Expression of tumor necrosis factor ligand superfamily costimulatory molecules CD27L, CD30L, OX40L and 4-1BBL in the heart of patients with acute myocarditis and dilated cardiomyopathy. *Cardiovasc Pathol* 2002; **11**: 166-170 [PMID: 12031769]
53 **Burgess JK**, Carlin S, Pack RA, Arndt GM, Au WW, Johnson PR, Black JL, Hunt NH. Detection and characterization of OX40 ligand expression in human airway smooth muscle cells: a possible role in asthma? *J Allergy Clin Immunol* 2004; **113**: 683-689 [PMID: 15100674 DOI: 10.1016/j.jaci.2003.12.311]
54 **Wing K**, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol* 2010; **11**: 7-13 [PMID: 20016504 DOI: 10.1038/ni.1818]
55 **Sakaguchi S**. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; **6**: 345-352 [PMID: 15785760 DOI: 10.1038/ni1178]
56 **Fontenot JD**, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 2005; **22**: 329-341 [PMID: 15780990 DOI: 10.1016/j.immuni.2005.01.016]
57 **Fontenot JD**, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; **4**: 330-336 [PMID: 12612578 DOI: 10.1038/ni904]
58 **Brunkow ME**, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 2001; **27**: 68-73 [PMID: 11138001 DOI: 10.1038/83784]
59 **Wildin RS**, Ramsdell F, Peake J, Faravelli F, Casanova JL,

Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli FD, Byrne G, McEuen M, Proll S, Appleby M, Brunkow ME. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 2001; **27**: 18-20 [PMID: 11137992 DOI: 10.1038/83707]

60 **Chen W**, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; **198**: 1875-1886 [PMID: 14676299 DOI: 10.1084/jem.20030152]

61 **Fantini MC**, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 2004; **172**: 5149-5153 [PMID: 15100250]

62 **Knoechel B**, Lohr J, Kahn E, Bluestone JA, Abbas AK. Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. *J Exp Med* 2005; **202**: 1375-1386 [PMID: 16287710 DOI: 10.1084/jem.20050855]

63 **Petrausch U**, Jensen SM, Twitty C, Poehlein CH, Haley DP, Walker EB, Fox BA. Disruption of TGF-beta signaling prevents the generation of tumor-sensitized regulatory T cells and facilitates therapeutic antitumor immunity. *J Immunol* 2009; **183**: 3682-3689 [PMID: 19692636 DOI: 10.4049/jimmunol.0900560]

64 **Sawamukai N**, Satake A, Schmidt AM, Lamborn IT, Ojha P, Tanaka Y, Kambayashi T. Cell-autonomous role of TGFβ and IL-2 receptors in CD4+ and CD8+ inducible regulatory T-cell generation during GVHD. *Blood* 2012; **119**: 5575-5583 [PMID: 22496155 DOI: 10.1182/blood-2011-07-367987]

65 **Maldonado RA**, von Andrian UH. How tolerogenic dendritic cells induce regulatory T cells. *Adv Immunol* 2010; **108**: 111-165 [PMID: 21056730 DOI: 10.1016/B978-0-12-380995-7.00004-5]

66 **Elias KM**, Laurence A, Davidson TS, Stephens G, Kanno Y, Shevach EM, O'Shea JJ. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* 2008; **111**: 1013-1020 [PMID: 17951529 DOI: 10.1182/blood-2007-06-096438]

67 **Xiao S**, Jin H, Korn T, Liu SM, Oukka M, Lim B, Kuchroo VK. Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J Immunol* 2008; **181**: 2277-2284 [PMID: 18684916]

68 **Bilate AM**, Lafaille JJ. Induced CD4+Foxp3+ regulatory T cells in immune tolerance. *Annu Rev Immunol* 2012; **30**: 733-758 [PMID: 22224762 DOI: 10.1146/annurev-immunol-020711-075043]

69 **Thornton AM**, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, Shevach EM. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 2010; **184**: 3433-3441 [PMID: 20181882 DOI: 10.4049/jimmunol.0904028]

70 **Verhagen J**, Wraith DC. Comment on "Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells". *J Immunol* 2010; **185**: 7129; author reply 7130 [PMID: 21127313 DOI: 10.4049/jimmunol.1090105]

71 **Akimova T**, Beier UH, Wang L, Levine MH, Hancock WW. Helios expression is a marker of T cell activation and proliferation. *PLoS One* 2011; **6**: e24226 [PMID: 21918685 DOI: 10.1371/journal.pone.0024226]

72 **Weiss JM**, Bilate AM, Gobert M, Ding Y, Curotto de Lafaille MA, Parkhurst CN, Xiong H, Dolpady J, Frey AB, Ruocco MG, Yang Y, Floess S, Huehn J, Oh S, Li MO, Niec RE, Rudensky AY, Dustin ML, Littman DR, Lafaille JJ. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med* 2012; **209**: 1723-1742, S1 [PMID: 22966001 DOI: 10.1084/jem.20120914]

73 **Groux H**, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997; **389**: 737-742 [PMID: 9338786 DOI: 10.1038/39614]

74 **Miller A**, Lider O, Roberts AB, Sporn MB, Weiner HL. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc Natl Acad Sci USA* 1992; **89**: 421-425 [PMID: 1370356]

75 **Weiner HL**. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* 2001; **182**: 207-214 [PMID: 11722636]

76 **Thornton AM**, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998; **188**: 287-296 [PMID: 9670041]

77 **Takahashi T**, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, Shimizu J, Sakaguchi S. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 1998; **10**: 1969-1980 [PMID: 9885918]

78 **Yamazaki S**, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K, Steinman RM. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* 2003; **198**: 235-247 [PMID: 12874257 DOI: 10.1084/jem.20030422]

79 **Tonkin DR**, He J, Barbour G, Haskins K. Regulatory T cells prevent transfer of type 1 diabetes in NOD mice only when their antigen is present in vivo. *J Immunol* 2008; **181**: 4516-4522 [PMID: 18802054]

80 **Wing K**, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 2008; **322**: 271-275 [PMID: 18845758 DOI: 10.1126/science.1160062]

81 **Takahashi T**, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 2000; **192**: 303-310 [PMID: 10899917]

82 **Qureshi OS**, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, Baker J, Jeffery LE, Kaur S, Briggs Z, Hou TZ, Futter CE, Anderson G, Walker LS, Sansom DM. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* 2011; **332**: 600-603 [PMID: 21474713 DOI: 10.1126/science.1202947]

83 **Munn DH**, Sharma MD, Mellor AL. Ligation of B7-1/B7-2 by human CD4+ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. *J Immunol* 2004; **172**: 4100-4110 [PMID: 15034022]

84 **Dejean AS**, Beisner DR, Ch'en IL, Kerdiles YM, Babour A, Arden KC, Castrillon DH, DePinho RA, Hedrick SM. Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells. *Nat Immunol* 2009; **10**: 504-513 [PMID: 19363483 DOI: 10.1038/ni.1729]

85 **Veldhoen M**, Moncrieffe H, Hocking RJ, Atkins CJ, Stockinger B. Modulation of dendritic cell function by naive and regulatory CD4+ T cells. *J Immunol* 2006; **176**: 6202-6210 [PMID: 16670330]

86 **Asseman C**, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 1999; **190**: 995-1004 [PMID: 10510089]

87 **Li MO**, Sanjabi S, Flavell RA. Transforming growth factor-

beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* 2006; **25**: 455-471 [PMID: 16973386 DOI: 10.1016/j.immuni.2006.07.011]

88 **Collison LW**, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 2007; **450**: 566-569 [PMID: 18033300 DOI: 10.1038/nature06306]

89 **Cao X**, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR, Ley TJ. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 2007; **27**: 635-646 [PMID: 17919943 DOI: 10.1016/j.immuni.2007.08.014]

90 **Andersson J**, Tran DQ, Pesu M, Davidson TS, Ramsey H, O'Shea JJ, Shevach EM. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. *J Exp Med* 2008; **205**: 1975-1981 [PMID: 18710931 DOI: 10.1084/jem.20080308]

91 **Bopp T**, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, Heib V, Becker M, Kubach J, Schmitt S, Stoll S, Schild H, Staege MS, Stassen M, Jonuleit H, Schmitt E. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 2007; **204**: 1303-1310 [PMID: 17502663 DOI: 10.1084/jem.20062129]

92 **Ernst PB**, Garrison JC, Thompson LF. Much ado about adenosine: adenosine synthesis and function in regulatory T cell biology. *J Immunol* 2010; **185**: 1993-1998 [PMID: 20686167 DOI: 10.4049/jimmunol.1000108]

93 **Pace KE**, Lee C, Stewart PL, Baum LG. Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1. *J Immunol* 1999; **163**: 3801-3811 [PMID: 10490978]

94 **Pandiyan P**, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* 2007; **8**: 1353-1362 [PMID: 17982458 DOI: 10.1038/ni1536]

95 **Yu A**, Zhu L, Altman NH, Malek TR. A low interleukin-2 receptor signaling threshold supports the development and homeostasis of T regulatory cells. *Immunity* 2009; **30**: 204-217 [PMID: 19185518 DOI: 10.1016/j.immuni.2008.11.014]

96 **Papiernik M**, de Moraes ML, Pontoux C, Vasseur F, Pénit C. Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. *Int Immunol* 1998; **10**: 371-378 [PMID: 9620592]

97 **Wing JB**, Sakaguchi S. Multiple treg suppressive modules and their adaptability. *Front Immunol* 2012; **3**: 178 [PMID: 22754556 DOI: 10.3389/fimmu.2012.00178]

98 **Read S**, Greenwald R, Izcue A, Robinson N, Mandelbrot D, Francisco L, Sharpe AH, Powrie F. Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. *J Immunol* 2006; **177**: 4376-4383 [PMID: 16982872]

99 **Suri-Payer E**, Cantor H. Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4(+)CD25(+) T cells. *J Autoimmun* 2001; **16**: 115-123 [PMID: 11247637 DOI: 10.1006/jaut.2000.0473]

100 **Vincenti F**. Costimulation blockade in autoimmunity and transplantation. *J Allergy Clin Immunol* 2008; **121**: 299-306; quiz 307-308 [PMID: 18269922 DOI: 10.1016/j.jaci.2008.01.002]

101 **Lenschow DJ**, Zeng Y, Thistlethwaite JR, Montag A, Brady W, Gibson MG, Linsley PS, Bluestone JA. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science* 1992; **257**: 789-792 [PMID: 1323143]

102 **Lin H**, Bolling SF, Linsley PS, Wei RQ, Gordon D, Thompson CB, Turka LA. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J Exp Med* 1993; **178**: 1801-1806 [PMID: 8228826]

103 **Blazar BR**, Taylor PA, Linsley PS, Vallera DA. In vivo blockade of CD28/CTLA4: B7/BB1 interaction with CTLA4-Ig reduces lethal murine graft-versus-host disease across the major histocompatibility complex barrier in mice. *Blood* 1994; **83**: 3815-3825 [PMID: 7515723]

104 **Pearson TC**, Alexander DZ, Winn KJ, Linsley PS, Lowry RP, Larsen CP. Transplantation tolerance induced by CTLA4-Ig. *Transplantation* 1994; **57**: 1701-1706 [PMID: 8016872]

105 **Cross AH**, Girard TJ, Giacoletto KS, Evans RJ, Keeling RM, Lin RF, Trotter JL, Karr RW. Long-term inhibition of murine experimental autoimmune encephalomyelitis using CTLA-4-Fc supports a key role for CD28 costimulation. *J Clin Invest* 1995; **95**: 2783-2789 [PMID: 7539461 DOI: 10.1172/JCI117982]

106 **Lenschow DJ**, Ho SC, Sattar H, Rhee L, Gray G, Nabavi N, Herold KC, Bluestone JA. Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. *J Exp Med* 1995; **181**: 1145-1155 [PMID: 7532678]

107 **Finck BK**, Linsley PS, Wofsy D. Treatment of murine lupus with CTLA4Ig. *Science* 1994; **265**: 1225-1227 [PMID: 7520604]

108 **Davies JK**, Gribben JG, Brennan LL, Yuk D, Nadler LM, Guinan EC. Outcome of alloanergized haploidentical bone marrow transplantation after ex vivo costimulatory blockade: results of 2 phase 1 studies. *Blood* 2008; **112**: 2232-2241 [PMID: 18617635 DOI: 10.1182/blood-2008-03-143636]

109 **Koura DT**, Horan JT, Langston AA, Qayed M, Mehta A, Khoury HJ, Harvey RD, Suessmuth Y, Couture C, Carr J, Grizzle A, Johnson HR, Cheeseman JA, Conger JA, Robertson J, Stempora L, Johnson BE, Garrett A, Kirk AD, Larsen CP, Waller EK, Kean LS. In vivo T cell costimulation blockade with abatacept for acute graft-versus-host disease prevention: a first-in-disease trial. *Biol Blood Marrow Transplant* 2013; **19**: 1638-1649 [PMID: 24047754 DOI: 10.1016/j.bbmt.2013.09.003]

110 **Genovese MC**, Becker JC, Schiff M, Luggen M, Sherrer Y, Kremer J, Birbara C, Box J, Natarajan K, Nuamah I, Li T, Aranda R, Hagerty DT, Dougados M. Abatacept for rheumatoid arthritis refractory to tumor necrosis factor alpha inhibition. *N Engl J Med* 2005; **353**: 1114-1123 [PMID: 16162882 DOI: 10.1056/NEJMoa050524]

111 **Charpentier B**. Belatacept: a novel immunosuppressive agent for kidney transplant recipients. *Expert Rev Clin Immunol* 2012; **8**: 719-728 [PMID: 23167683 DOI: 10.1586/eci.12.79]

112 **Blazar BR**, Taylor PA, Panoskaltsis-Mortari A, Buhlman J, Xu J, Flavell RA, Korngold R, Noelle R, Vallera DA. Blockade of CD40 ligand-CD40 interaction impairs CD4+ T cell-mediated alloreactivity by inhibiting mature donor T cell expansion and function after bone marrow transplantation. *J Immunol* 1997; **158**: 29-39 [PMID: 8977172]

113 **Larsen CP**, Alexander DZ, Hollenbaugh D, Elwood ET, Ritchie SC, Aruffo A, Hendrix R, Pearson TC. CD40-gp39 interactions play a critical role during allograft rejection. Suppression of allograft rejection by blockade of the CD40-gp39 pathway. *Transplantation* 1996; **61**: 4-9 [PMID: 8560571]

114 **Bumgardner GL**, Li J, Heininger M, Orosz CG. Costimulation pathways in host immune responses to allogeneic hepatocytes. *Transplantation* 1998; **66**: 1841-1845 [PMID: 9884287]

115 **Molano RD**, Berney T, Li H, Cattan P, Pileggi A, Vizzardelli C, Kenyon NS, Ricordi C, Burkly LC, Inverardi L. Prolonged islet graft survival in NOD mice by blockade of the CD40-CD154 pathway of T-cell costimulation. *Diabetes* 2001; **50**: 270-276 [PMID: 11272136]

116 **Tung TH**, Mackinnon SE, Mohanakumar T. Long-term limb allograft survival using anti-CD40L antibody in a murine model. *Transplantation* 2003; **75**: 644-650 [PMID: 12640303 DOI: 10.1097/01.TP.0000053756.90975.8E]

117 **Law CL**, Grewal IS. Therapeutic interventions targeting CD40L (CD154) and CD40: the opportunities and challenges. *Adv Exp Med Biol* 2009; **647**: 8-36 [PMID: 19760064 DOI: 10.1007/978-0-387-89520-8_2]

118 **Sidiropoulos PI**, Boumpas DT. Lessons learned from anti-

CD40L treatment in systemic lupus erythematosus patients. *Lupus* 2004; **13**: 391-397 [PMID: 15230298]
119 CDP7657, a Monovalent Fab PEG Anti-CD40L Antibody, Inhibits Immune Responses in Both HuSCID Mice and Non-Human Primates. *Arthritis Rheum* 2010: Suppl 10: 1245 [DOI: 10.1002/art.29011]
120 **Pinelli DF**, Wagener ME, Liu D, Yamniuk A, Tamura J, Grant S, Larsen CP, Suri A, Nadler SG, Ford ML. An anti-CD154 domain antibody prolongs graft survival and induces Foxp3(+) iTreg in the absence and presence of CTLA-4 Ig. *Am J Transplant* 2013; **13**: 3021-3030 [PMID: 24007441 DOI: 10.1111/ajt.12417]
121 **Daley SR**, Cobbold SP, Waldmann H. Fc-disabled anti-mouse CD40L antibodies retain efficacy in promoting transplantation tolerance. *Am J Transplant* 2008; **8**: 2265-2271 [PMID: 18782294 DOI: 10.1111/j.1600-6143.2008.02382.x]
122 **Margolles-Clark E**, Umland O, Kenyon NS, Ricordi C, Buchwald P. Small-molecule costimulatory blockade: organic dye inhibitors of the CD40-CD154 interaction. *J Mol Med* (Berl) 2009; **87**: 1133-1143 [PMID: 19707732 DOI: 10.1007/s00109-009-0519-3]
123 **Allen SD**, Rawale SV, Whitacre CC, Kaumaya PT. Therapeutic peptidomimetic strategies for autoimmune diseases: costimulation blockade. *J Pept Res* 2005; **65**: 591-604 [PMID: 15885118 DOI: 10.1111/j.1399-3011.2005.00256.x]
124 **Tai YT**, Li X, Tong X, Santos D, Otsuki T, Catley L, Tournilhac O, Podar K, Hideshima T, Schlossman R, Richardson P, Munshi NC, Luqman M, Anderson KC. Human anti-CD40 antagonist antibody triggers significant antitumor activity against human multiple myeloma. *Cancer Res* 2005; **65**: 5898-5906 [PMID: 15994968 DOI: 10.1158/0008-5472.CAN-04-4125]
125 **Luqman M**, Klabunde S, Lin K, Georgakis GV, Cherukuri A, Holash J, Goldbeck C, Xu X, Kadel EE, Lee SH, Aukerman SL, Jallal B, Aziz N, Weng WK, Wierda W, O'Brien S, Younes A. The antileukemia activity of a human anti-CD40 antagonist antibody, HCD122, on human chronic lymphocytic leukemia cells. *Blood* 2008; **112**: 711-720 [PMID: 18497318 DOI: 10.1182/blood-2007-04-084756]
126 **Byrd JC**, Kipps TJ, Flinn IW, Cooper M, Odenike O, Bendiske J, Rediske J, Bilic S, Dey J, Baeck J, O'Brien S. Phase I study of the anti-CD40 humanized monoclonal antibody lucatumumab (HCD122) in relapsed chronic lymphocytic leukemia. *Leuk Lymphoma* 2012; **53**: 2136-2142 [PMID: 22475052 DOI: 10.3109/10428194.2012.681655]
127 **Boon L**, Brok HP, Bauer J, Ortiz-Buijsse A, Schellekens MM, Ramdien-Murli S, Blezer E, van Meurs M, Ceuppens J, de Boer M, 't Hart BA, Laman JD. Prevention of experimental autoimmune encephalomyelitis in the common marmoset (Callithrix jacchus) using a chimeric antagonist monoclonal antibody against human CD40 is associated with altered B cell responses. *J Immunol* 2001; **167**: 2942-2949 [PMID: 11509643]
128 **Kasran A**, Boon L, Wortel CH, Hogezand RA, Schreiber S, Goldin E, Boer M, Geboes K, Rutgeerts P, Ceuppens JL. Safety and tolerability of antagonist anti-human CD40 Mab ch5D12 in patients with moderate to severe Crohn's disease. *Aliment Pharmacol Ther* 2005; **22**: 111-122 [PMID: 16011669 DOI: 10.1111/j.1365-2036.2005.02526.x]
129 **Kopf M**, Coyle AJ, Schmitz N, Barner M, Oxenius A, Gallimore A, Gutierrez-Ramos JC, Bachmann MF. Inducible costimulator protein (ICOS) controls T helper cell subset polarization after virus and parasite infection. *J Exp Med* 2000; **192**: 53-61 [PMID: 10880526]
130 **Howland KC**, Ausubel LJ, London CA, Abbas AK. The roles of CD28 and CD40 ligand in T cell activation and tolerance. *J Immunol* 2000; **164**: 4465-4470 [PMID: 10779746]
131 **Larsen CP**, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, Cho HR, Aruffo A, Hollenbaugh D, Linsley PS, Winn KJ, Pearson TC. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 1996; **381**: 434-438 [PMID: 8632801 DOI: 10.1038/381434a0]
132 **Kirk AD**, Harlan DM, Armstrong NN, Davis TA, Dong Y, Gray GS, Hong X, Thomas D, Fechner JH, Knechtle SJ. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci USA* 1997; **94**: 8789-8794 [PMID: 9238056]
133 **Ito D**, Ogasawara K, Iwabuchi K, Inuyama Y, Onoé K. Induction of CTL responses by simultaneous administration of liposomal peptide vaccine with anti-CD40 and anti-CTLA-4 mAb. *J Immunol* 2000; **164**: 1230-1235 [PMID: 10640735]
134 **Schaub M**, Issazadeh S, Stadlbauer TH, Peach R, Sayegh MH, Khoury SJ. Costimulatory signal blockade in murine relapsing experimental autoimmune encephalomyelitis. *J Neuroimmunol* 1999; **96**: 158-166 [PMID: 10337914]
135 **Wang X**, Huang W, Mihara M, Sinha J, Davidson A. Mechanism of action of combined short-term CTLA4Ig and anti-CD40 ligand in murine systemic lupus erythematosus. *J Immunol* 2002; **168**: 2046-2053 [PMID: 11823542]
136 **Verbinnen B**, Billiau AD, Vermeiren J, Galicia G, Bullens DM, Boon L, Cadot P, Hens G, Dewolf-Peeters C, Van Gool SW, Ceuppens JL. Contribution of regulatory T cells and effector T cell deletion in tolerance induction by costimulation blockade. *J Immunol* 2008; **181**: 1034-1042 [PMID: 18606655]
137 **Wekerle T**, Sachs DH, Sykes M. Mixed chimerism for the induction of tolerance: potential applicability in clinical composite tissue grafting. *Transplant Proc* 1998; **30**: 2708-2710 [PMID: 9745551]
138 **Li Y**, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat Med* 1999; **5**: 1298-1302 [PMID: 10545997 DOI: 10.1038/15256]
139 **Wells AD**, Li XC, Li Y, Walsh MC, Zheng XX, Wu Z, Nuñez G, Tang A, Sayegh M, Hancock WW, Strom TB, Turka LA. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med* 1999; **5**: 1303-1307 [PMID: 10545998 DOI: 10.1038/15260]
140 **Li XC**, Li Y, Dodge I, Wells AD, Zheng XX, Turka LA, Strom TB. Induction of allograft tolerance in the absence of Fas-mediated apoptosis. *J Immunol* 1999; **163**: 2500-2507 [PMID: 10452986]
141 **Grohmann U**, Orabona C, Fallarino F, Vacca C, Calcinaro F, Falorni A, Candeloro P, Belladonna ML, Bianchi R, Fioretti MC, Puccetti P. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat Immunol* 2002; **3**: 1097-1101 [PMID: 12368911 DOI: 10.1038/ni846]
142 **Kurtz J**, Ito H, Wekerle T, Shaffer J, Sykes M. Mechanisms involved in the establishment of tolerance through costimulatory blockade and BMT: lack of requirement for CD40L-mediated signaling for tolerance or deletion of donor-reactive CD4+ cells. *Am J Transplant* 2001; **1**: 339-349 [PMID: 12099378]
143 **Honey K**, Cobbold SP, Waldmann H. CD40 ligand blockade induces CD4+ T cell tolerance and linked suppression. *J Immunol* 1999; **163**: 4805-4810 [PMID: 10528180]
144 **Graca L**, Honey K, Adams E, Cobbold SP, Waldmann H. Cutting edge: anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *J Immunol* 2000; **165**: 4783-4786 [PMID: 11045999]
145 **Taylor PA**, Noelle RJ, Blazar BR. CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med* 2001; **193**: 1311-1318 [PMID: 11390438]
146 **Quezada SA**, Bennett K, Blazar BR, Rudensky AY, Sakaguchi S, Noelle RJ. Analysis of the underlying cellular mechanisms of anti-CD154-induced graft tolerance: the interplay of clonal anergy and immune regulation. *J Immunol* 2005; **175**: 771-779 [PMID: 16002673]

147 **Jiang X**, Sun W, Guo D, Cui Z, Zhu L, Lin L, Tang Y, Wang X, Liang J. Cardiac allograft acceptance induced by blockade of CD40-CD40L costimulation is dependent on CD4+CD25+ regulatory T cells. *Surgery* 2011; **149**: 336-346 [PMID: 20875655 DOI: 10.1016/j.surg.2010.08.012]

148 **Kurtz J**, Shaffer J, Lie A, Anosova N, Benichou G, Sykes M. Mechanisms of early peripheral CD4 T-cell tolerance induction by anti-CD154 monoclonal antibody and allogeneic bone marrow transplantation: evidence for anergy and deletion but not regulatory cells. *Blood* 2004; **103**: 4336-4343 [PMID: 14962909 DOI: 10.1182/blood-2003-08-2642]

149 **Tang Q**, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, Zheng XX, Strom TB, Bluestone JA. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 2003; **171**: 3348-3352 [PMID: 14500627]

150 **Salomon B**, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 2000; **12**: 431-440 [PMID: 10795741]

151 **Pacholczyk R**, Kern J. The T-cell receptor repertoire of regulatory T cells. *Immunology* 2008; **125**: 450-458 [PMID: 19128356 DOI: 10.1111/j.1365-2567.2008.02992.x]

152 **Martín-Gayo E**, Sierra-Filardi E, Corbí AL, Toribio ML. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood* 2010; **115**: 5366-5375 [PMID: 20357241 DOI: 10.1182/blood-2009-10-248260]

153 **McHugh RS**, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, Byrne MC. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 2002; **16**: 311-323 [PMID: 11869690]

154 **Vermeiren J**, Ceuppens JL, Van Ghelue M, Witters P, Bullens D, Mages HW, Kroczek RA, Van Gool SW. Human T cell activation by costimulatory signal-deficient allogeneic cells induces inducible costimulator-expressing anergic T cells with regulatory cell activity. *J Immunol* 2004; **172**: 5371-5378 [PMID: 15100277]

155 **Vogel I**, Verbinnen B, Maes W, Boon L, Van Gool SW, Ceuppens JL. Foxp3+ regulatory T cells are activated in spite of B7-CD28 and CD40-CD40L blockade. *Eur J Immunol* 2013; **43**: 1013-1023 [PMID: 23348953 DOI: 10.1002/eji.201242737]

156 **Coenen JJ**, Koenen HJ, van Rijssen E, Hilbrands LB, Joosten I. Tolerizing effects of co-stimulation blockade rest on functional dominance of CD4+CD25+ regulatory T cells. *Transplantation* 2005; **79**: 147-156 [PMID: 15665762]

157 **Poirier N**, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, Tillou X, Wu G, Reneaudin K, Hervouet J, Martinet B, Coulon F, Allain-Launay E, Karam G, Soulillou JP, Pierson RN, Blancho G, Vanhove B. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. *Sci Transl Med* 2010; **2**: 17ra10 [PMID: 20371478 DOI: 10.1126/scitranslmed.3000116]

158 **Igarashi H**, Cao Y, Iwai H, Piao J, Kamimura Y, Hashiguchi M, Amagasa T, Azuma M. GITR ligand-costimulation activates effector and regulatory functions of CD4+ T cells. *Biochem Biophys Res Commun* 2008; **369**: 1134-1138 [PMID: 18346459 DOI: 10.1016/j.bbrc.2008.03.024]

159 **Akbari O**, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, Berry G, DeKruyff RH, Umetsu DT. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med* 2002; **8**: 1024-1032 [PMID: 12145647 DOI: 10.1038/nm745]

160 **Wang C**, Li Y, Proctor TM, Vandenbark AA, Offner H. Down-modulation of programmed death 1 alters regulatory T cells and promotes experimental autoimmune encephalomyelitis. *J Neurosci Res* 2010; **88**: 7-15 [PMID: 19642196 DOI: 10.1002/jnr.22181]

161 **Verhagen J**, Gabrysová L, Minaee S, Sabatos CA, Anderson G, Sharpe AH, Wraith DC. Enhanced selection of FoxP3+ T-regulatory cells protects CTLA-4-deficient mice from CNS autoimmune disease. *Proc Natl Acad Sci USA* 2009; **106**: 3306-3311 [PMID: 19218450 DOI: 10.1073/pnas.0803186106]

162 **Tang AL**, Teijaro JR, Njau MN, Chandran SS, Azimzadeh A, Nadler SG, Rothstein DM, Farber DL. CTLA4 expression is an indicator and regulator of steady-state CD4+ FoxP3+ T cell homeostasis. *J Immunol* 2008; **181**: 1806-1813 [PMID: 18641318]

163 **Yamaguchi T**, Kishi A, Osaki M, Morikawa H, Prieto-Martin P, Wing K, Saito T, Sakaguchi S. Construction of self-recognizing regulatory T cells from conventional T cells by controlling CTLA-4 and IL-2 expression. *Proc Natl Acad Sci USA* 2013; **110**: E2116-E2125 [PMID: 23690575 DOI: 10.1073/pnas.1307185110]

164 **Barnes MJ**, Griseri T, Johnson AM, Young W, Powrie F, Izcue A. CTLA-4 promotes Foxp3 induction and regulatory T cell accumulation in the intestinal lamina propria. *Mucosal Immunol* 2013; **6**: 324-334 [PMID: 22910217 DOI: 10.1038/mi.2012.75]

165 **Xiao X**, Gong W, Demirci G, Liu W, Spoerl S, Chu X, Bishop DK, Turka LA, Li XC. New insights on OX40 in the control of T cell immunity and immune tolerance in vivo. *J Immunol* 2012; **188**: 892-901 [PMID: 22147766 DOI: 10.4049/jimmunol.1101373]

166 **Li W**, Carlson TL, Green WR. Stimulation-dependent induction of CD154 on a subset of CD4+ FoxP3+ T-regulatory cells. *Int Immunopharmacol* 2011; **11**: 1205-1210 [PMID: 21496498 DOI: 10.1016/j.intimp.2011.03.021]Available]

167 **Riella LV**, Liu T, Yang J, Chock S, Shimizu T, Mfarrej B, Batal I, Xiao X, Sayegh MH, Chandraker A. Deleterious effect of CTLA4-Ig on a Treg-dependent transplant model. *Am J Transplant* 2012; **12**: 846-855 [PMID: 22300534 DOI: 10.1111/j.1600-6143.2011.03929.x]

168 **Charbonnier LM**, Vokaer B, Lemaître PH, Field KA, Leo O, Le Moine A. CTLA4-Ig restores rejection of MHC class-II mismatched allografts by disabling IL-2-expanded regulatory T cells. *Am J Transplant* 2012; **12**: 2313-2321 [PMID: 22759373 DOI: 10.1111/j.1600-6143.2012.04184.x]

169 **Tai X**, Van Laethem F, Pobezinsky L, Guinter T, Sharrow SO, Adams A, Granger L, Kruhlak M, Lindsten T, Thompson CB, Feigenbaum L, Singer A. Basis of CTLA-4 function in regulatory and conventional CD4(+) T cells. *Blood* 2012; **119**: 5155-5163 [PMID: 22403258 DOI: 10.1182/blood-2011-11-388918]

P- Reviewer: Camara NOS, Chui YL, Haque A **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Wang CH

World Journal of Immunology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5411/wji.v4.i2.78

World J Immunol 2014 July 27; 4(2): 78-87
ISSN 2219-2824 (online)

REVIEW

Immunopathogenesis of reactive arthritis: Role of the cytokines

Ricardo Javier Eliçabe, María Silvia Di Genaro

Ricardo Javier Eliçabe, María Silvia Di Genaro, Division of Immunology, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, San Luis 5700, Argentine
Ricardo Javier Eliçabe, María Silvia Di Genaro, Laboratory of Immunopathology, Multidisciplinary Institute of Biological Investigations San Luis, San Luis 5700, Argentine
Author contributions: Eliçabe RJ and Di Genaro MS designed the review and wrote the paper; Eliçabe RJ made the figure and contributed to the editing of the paper.
Supported by Agencia Nacional de Promoción Científica y Tecnológica, No. PICT 2008-763, PICT 2011-732; by the National University of San Luis (Project 0401); by the Scientific Career of National Council of Scientific and Technical Investigations; and by National Council of Scientific and Technical Investigations
Correspondence to: María Silvia Di Genaro, Professor, PhD, Laboratory of Immunopathology, Multidisciplinary Institute of Biological Investigations San Luis, 950 Ejército de los Andes Street, San Luis 5700, Argentina. sdigena@unsl.edu.ar
Telephone: +54-0266-4520300 Fax: +54-0266-4422644
Received: March 15, 2014 Revised: May 24, 2014
Accepted: June 14, 2014
Published online: July 27, 2014

Abstract

Reactive arthritis (ReA), also known as sterile postinfectious arthritis, belongs to the group of related arthropathies known as spondyloarthritis (SpA). ReA can arise 1-4 wk after a gastrointestinal or genitourinary infection, but once arthritis develops, the microorganism is not found in the joint. The classical microbes associated with ReA development include Gram-negative aerobic or microaerophilic bacteria containing LPS in their outer membrane. The immunopathogenic mechanisms involved in ReA development are still unknown. A hypothesis suggested that the bacteria probably persist outside the joint, at sites such as gut mucosa or lymph nodes, and bacterial antigens might then be transported to the joints. On the other hand, an altered immune response and the unbalanced production of cytokines have been reported in subjects with ReA. Currently, there is increased evidence to suggest that both mechanisms would operate in the immunopathogenesis of ReA. In this review we highlight recent advances on the role of cytokines in the ReA. Particularly, we discuss the roles of some pro- and anti-inflammatory cytokines involved in the immunopathogenesis of ReA.

Key words: Reactive arthritis; Cytokines; Immunopathogenesis; Infection; Interleukin-17; Interleukin-12; Interleukin-23; Interleukin-6; Tumor necrosis factor-α; Interleukin-10

Core tip: The immunopathogenic mechanisms involved in reactive arthritis (ReA) development are still unknown. However, in the last years, increased evidence suggests that the immune response in particular certain cytokines could be involved in the pathogenesis of ReA. Currently, the use of biological agents that block the action of certain cytokines has contributed to improving the treatment of some rheumatic pathology. Understanding the role of cytokines in the pathogenesis of ReA could contribute to the development of future treatments. In this review, we highlight recent advances on the role of certain cytokines in the pathogenesis of ReA.

Eliçabe RJ, Di Genaro MS. Immunopathogenesis of reactive arthritis: Role of the cytokines. *World J Immunol* 2014; 4(2): 78-87 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i2/78.htm DOI: http://dx.doi.org/10.5411/wji.v4.i2.78

INTRODUCTION

Reactive arthritis (ReA), also known as sterile postinfectious arthritis, belongs to the group of related arthropathies known as spondyloarthritis (SpA)[1]. This group

also includes undifferentiated SpA, psoriatic arthritis (PsA), arthritis associated with inflammatory bowel disease and ankylosing spondylitis (AS). The SpA arthropathies have common several epidemiological, pathological, clinical and radiological features. ReA, as with other SpA, exhibits an absence of rheumatoid factor and has a genetic association with the molecule HLA-B27[1-3]. ReA can arise 1-4 wk after a gastrointestinal or genitourinary infection, but once arthritis develops, the microorganism is not found in the joint[2]. The ReA symptoms were recognized and studied in 1942 by Bauer and Engelmann, who associated these symptoms with those described in 1916 by the German physician Hans Reiter. At that time, Reiter described the clinical triad: arthritis, nongonococcal urethritis and conjunctivitis in a German soldier after an episode of bloody diarrhea. So, Bauer and Engelmann coined the term Reiter's syndrome to describe this new pathology[2]. However, most patients do not have the complete triad of symptoms. These observations drove Ahvonen to propose the name of ReA as the term most adapted to describe the "arthritis that happens during or after an infection in another site of the body without evidence of microorganisms in the joint"[4]. Yet, this operational definition of ReA has led to uncertain diagnosis in different clinical settings. Thus, several attempts have been made to create classification criteria; however, lack of consensus has led to a failure to achieve any universally validated diagnostic criteria. Based on discussions at the 4th International Workshop on ReA, this term should be used only in patients with clinical features of ReA and in cases where a pathogen known to cause ReA is implicated[5].

CLINICAL FEATURES

ReA most commonly affects young adults aged 20 to 40 years old and is rare in children[6-8]. Both sexes are equally affected by ReA after a gastrointestinal infection, while ReA is more frequent in men when triggered by a urogenital tract infection[3]. The presence of the HLA-B27 allele does not seem to be related to the onset of ReA; however, HLA-B27 positive patients have more severe arthritis with a tendency to progress to a chronic stage and they also have a greater chance of developing extra-articular symptoms. One hypothesis suggests that this molecule favors the cross-reaction between antigen and host, or it might be itself a target of the immune response[9].

The symptoms of ReA typically start between 1 to 4 wk after the gastrointestinal infection. However, the triggering infection could be asymptomatic, such as *Chlamydia*-induced ReA, resulting in underdiagnosis[2]. Clinical features of ReA are characterized by asymmetrical oligoarthritis, often in large joints of the lower extremities or in the upper extremities. A mild polyarticular form, particularly in the small joints, can also occur. Patients can have dactylitis. The typical extra-articular manifestations are enthesitis, tendinitis and bursitis. ReA share these clinical characteristics and inflammatory back pain with other members of SpA, such as AS and PsA[1]. Other extra-articular features include eye disease, where conjunctivitis is most prevalent, followed by acute anterior uveitis, and skin changes, such as erythema nodosum, keratodermia blennorrhagica and circinate balanitis[3].

The clinical diagnosis is made based on the clinical symptoms. Evidence for infection triggering the arthropathy is most convincing when microbe isolation or antigen detection is successful. In this respect, fecal culture of enteric pathogens associated with ReA or the finding of *Chlamydia trachomatis* nucleic acids in urine, cervical or urethral swabs are secondary criteria used to confirm the diagnosis.

Animal models

Animal models of ReA have complemented studies in human materials. However, these animal models are limited since even when they are developed after bacterial infection as in human ReA, in some of them the route of infection was intravenous instead of oral. Table 1 shows animal models of ReA similar to the human form of the disease[10-17]. We have described an experimental model useful for studying the pathogenesis of *Yersinia enterocolitica* (*Y. enterocolitica*) ReA. In our model, TNFRp55 deficient mice develop ReA after oral infection with *Y. enterocolitica* O: 3, the most common serotype associated with human ReA. *TNFRp55*$^{-/-}$ mice exhibited macroscopic signs of severe and progressive arthritis with significantly higher clinical score compared with wild-type mice from d 14 to 56 after infection[14]. Extensively, increased scores for inflammation and bone/cartilage degradation resulted when histopathological changes were analyzed in the joints. In these animals, we observed luminal disorganization of the synovial membrane, which was densely infiltrated with various types of leucocytes, sometimes concomitant with follicle formation. The articular cartilage and bone were degraded. Proliferation of synovial lining cells was also detected[14,15]. This evidence and the data presented in Table 1 indicate ReA development in animal models that resemble this disease in humans. Nevertheless, the convergence of these models with human studies will contribute to understand the pathogenic mechanisms of ReA.

TRIGGERING BACTERIAL AND PATHOPHYSIOLOGY

The classical bacteria associated with gastrointestinal ReA are *Yersinia*, *Salmonella*, *Shigella* and *Campylobacter*, while *C. trachomatis* is by far the most common cause of ReA associated with genital infection[3,18]. All these pathogens are Gram-negative aerobic or microaerophilic bacteria containing LPS in their outer membrane.

The immunopathogenic mechanisms involved in ReA development are still unknown. Even when bacterial

Table 1 Animal models of reactive arthritis similar to the human form of the disease

Animal	Bacteria	Route of infection	Arthritis onset/ remission	Clinical symptoms	Cytokine involved	Ref.
Lewis rats	*Y. enterocolitica* O:8[1]	*iv*[1]	1 wk/6 wk	Polyarticular arthritis, erythema	ND	Hill *et al*[10]
DBA/2 and BDF1 mice	*Y. enterocolitica* O:8 plasmid cured[1]	*iv*[1]	Day 31/3 wk	Polyarticular arthritis	ND	Yong *et al*[11]
SHR rats	*Y. enterocolitica* O:8[1]	*iv*[1]	1-4 wk/7-25 wk	Polyarticular arthritis, erythema, swelling and impaired movement of the joint	ND	Merilahti-Palo *et al*[12]
Swiss, BALB/c and C3H/HeJ mice	*Y. enterocolitica* O:3	*iv*[1]/*Oral*	1-3 wk/2-8 mo	Monoarticular arthritis, swelling redness, deformations and conjunctivitis	ND	de los Toyos *et al*[13]
C57BL/6 *TNFRp55*$^{-/-}$ mice	*Y. enterocolitica* O:3	*ig*	2 wk/chronic until 8 wk	Polyarticular arthritis, swelling, erythema	IL-17 IFN-γ IL-6 IL-1β	Di Genaro *et al*[14] Eliçabe *et al*[15]
BALB/c mice	*S. enteritides*	*ig*	1 wk/ND	Synovial inflammation	TNF-α IL-17	Noto Llana *et al*[16] Noto Llana *et al*[17]

[1]Different to the human form of the disease. ND: Not determined; *iv*: Intravenous infection; *ig*: Intragastric infection; IL: Interleukin; TNF: Tumor necrosis factor.

cultures of synovial fluids are negative in ReA, bacterial antigens have been found in the joints of patients. In *Chlamydia*-induced ReA, bacterial DNA and RNA have been detected in the joint, suggesting that live *Chlamydia* are present[19-21]. Positive reaction of antibodies specific to *Salmonella* and *Yersinia* antigens in synovial fluid cells of ReA patients suggests the presence of bacterial antigen in the joint[22,23]. Based on these findings, some authors have suggested that the bacteria probably persist outside the joint at sites such as gut mucosa or lymph nodes, and bacterial antigens might then be transported by monocytes to the joints[24,25]. On the other hand, an altered immune response and the unbalanced production of cytokines have been reported in subjects with ReA[26,27]. This altered immune response benefits the bacterial persistence and disfavors the elimination of the antigen by the host.

In this review, we highlight recent advances on the role of cytokines in ReA. Particularly, we discuss the roles of pro- and anti-inflammatory cytokines, especially interleukin (IL)-17, IL-12, IL-23, IL-6, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) as well as IL-10 in the pathophysiology of the ReA. Finally, we discuss the latest advances in the treatment of ReA based on the use of biological agents that neutralize the functions of certain cytokines, such as TNF-α or IL-6.

ROLE OF THE CYTOKINES IN ReA

Conflicting data have been reported on the production of cytokines in ReA patients. $CD4^+$ T cells mediate immunity as a balance between different lineages of T helper (Th)-1, Th2, Th3 and Th17 which secrete IFN-γ, IL-4, TGF-γ and IL-17, respectively, as the main cytokine for each profile. Some studies revealed low levels of Th1 cytokines in ReA, especially of TNF-α but also of IFN-γ in peripheral blood and synovium[28-34]. Since Th1 cells secreting IFN-γ and TNF-α have been proposed for bacterial clearance, defective Th1 response may contribute to bacterial persistence. Other data suggest that a Th2 cytokine profile and Th3 response with expression of TGF-β is common in ReA[32]. Temporal relationships of these different Th1 and Th2 cytokines or blunting of initial cytokine response might also be important in the disease manifestations and its maintenance. On the other hand, the discovery of Th17 cells and their importance in the pathogenesis of chronic inflammatory diseases suggested that these cells may have a pathogenic role in ReA. However, the available studies are not large enough to support the role of certain cytokines in the pathogenesis of ReA.

NOVEL CYTOKINES IMPLICATED IN PATHOGENESIS OF ReA

IL-17

IL-17 is a 15-20-kDa glycoprotein produced by a novel subset of Th cells, termed Th17 cells, and to a lesser extent by innate lymphoid cells, including T-cells, innate-like lymphoid cells, mast cells and neutrophils[35]. Th17 cells are critical in the pathogenesis of the arthritis, as demonstrated in several animal models[36-38]. Th17 differentiation, survival and expansion depend on a variety of cytokines and transcription factors that work in concert to drive the induction of increased Th17 numbers. TGF-β in synergy with IL-6 has been described as the central factor involved in generating Th17 cells in mice. It has been shown in humans that TGF-β, IL-1β and IL-6, combined with IL-21 or IL-23, can induce Th17 differentiation[39]. IL-17 binds to IL-17RA/IL-17RC, which is expressed by a variety of cells, such as monocytes, lymphocytes, lymphoid tissue inducer cells, epithelial cells, synoviocytes, fibroblasts and keratinocytes[35].

Th17 cell responses and IL-17 expression provide protection against bacterial and fungal pathogens through production and induction of inflammatory cytokines

and granulopoiesis, or by the recruitment of neutrophils. However, Th17 cells producing IL-17 have been suggested as the central effector lineage involved in the pathogenicity of ReA[40]. Thus, it has been shown that ReA patients have elevated levels of IL-17 in synovial fluid and that this cytokine contributes to the development of joint inflammation[40,41]. Furthermore, high expression of IL-17 was found in the synovial fluid of patients with SpA and an increased number of circulating memory Th17 cells has been recently reported in these patients[42,43]. Moreover, in patients with *C. trachomatis*-induced ReA, increased percentages of IL-17-positive $CD4^+$ T cells[44] and higher IL-17 concentrations were detected in synovial fluid[45].

Recent works suggest that *Salmonella*-induced ReA in mice dependent on $CD4^+$ T cells secreting IL-17[17]. Interestingly, these authors observed that the expression of IL-17 in the large intestine and in mesenteric lymph nodes (MLN) resembles that of popliteal and inguinal lymph nodes (ILN)[17]. Accordingly, previous results from our laboratory demonstrated that IL-17 plays a major role in *Yersinia*-induced ReA[15]. Furthermore, we detected a strong correlation among IL-17 levels in MLNs, ILNs and joints from *TNFRp55*$^{-/-}$ mice with arthritis, supporting a link between the intestinal mucosa and the articular immune response. In addition, we observed that neutralization of IL-17 resulted in the abrogation of synovitis[15]. In line with these results, other authors have reported recently that modulating intestinal IL-23/IL-17 expression by consumption of *Lactobacillus casei* prior to *Salmonella* infection in mice abolishes intestinal and joint inflammation[46].

These data in animal models and patients support the hypothesis that Th17 cells may be involved in ReA pathogenesis. However, there are few reports for understanding and elucidating the true role of IL-17 in the pathogenesis of ReA.

IL-12 and IL-23

IL-12 and IL-23 are heterodimeric cytokines that share subunits and have important roles in autoimmunity. These IL-12 family cytokines share some biological characteristics but have functional differences. IL-12 is composed of two covalently linked subunits, IL-12p35 and IL-12p40, while IL-23 is composed of two covalently linked subunits, IL-23p19, which is distantly related to IL-12p35, and the IL-12p40 subunit[47,48]. Furthermore, the receptors of IL-23 and IL-12 are also heterodimers that share the receptor 1 chain and have unique 2 chains[49]. IL-12 is released by antigen presenting cells such as dendritic cells (DCs) and monocytes/macrophages in response to bacterial products and immune signals. Furthermore, IL-12 is the main stimulator of IFN-γ production by inducing development of Th1 responses[49,50]. In addition, IL-23 is produced by macrophages and activated DCs and plays a crucial role in the generation of the Th17 cells. Since IL-12 has the ability to orchestrate the Th1 response, this cytokine plays a crucial role in the protective immunity against many pathogens associated with ReA. Thus, the low concentrations of IL-12 have been linked to the bacterial persistence hypothesis and then to the pathogenesis of ReA[28]. On the other hand, data on IL-23 concentrations in synovial fluid or serum of patients with ReA are limited, but high levels of IL-17 found in synovial fluids and sera of patients with ReA may reflect IL-23 activity. Moreover, abnormality of IL-12p40 gene expression in humans has been reported and IL-12 deficiency has been detected in patients with ReA[51,52]. Yin *et al*[28] found that the balance of anti-inflammatory cytokines (IL-10) and IL-12 in the synovial fluid is also important. This may contribute to the decreased clearance of the bacteria or their components from the joint and lead to ReA[28]. In relation to these findings, a recent study has shown that monocyte-derived macrophages from subjects with a history of ReA show low IL-12 and IL-23 production[53]. Conversely, some authors have reported that IL-12/23p40 levels in synovial fluids of patients with ReA and other SpA are higher compared to synovial fluids of patients with osteoarthritis (OA) used as control[41,54].

Interestingly, we demonstrated that the p40-deficient mice develop acute ReA after oral infection with *Y. enterocolitica*, suggesting that IL-12 or IL-23 could exert a protective effect on the development of ReA[55]. However, we have observed elevated levels of p40 in regional lymph nodes to joints of *TNFRp55*$^{-/-}$ mice with *Yersinia*-induced ReA. This effect has been accompanied by high levels of IFN-γ and IL-17 in affected joints[15]. These results are in accordance with the concept that the IL-12/IL-23 pathway plays a dual role protecting from infection and eliciting tissue damage, and support future study to determine whether IL-12/23p40 could be a possible target for ReA treatment.

IL-6

IL-6 is a pleiotropic cytokine that is involved in numerous biological processes. The pleiotropy and redundancy of IL-6 functions have been identified by characterizing a unique receptor system comprising two functional proteins: a receptor specific for IL-6 (IL-6R)[56] and gp130, the common signal transducer of cytokines related to IL-6, including the IL-12 family cytokines IL-27 and IL-35[57,58]. In the early phase of infectious inflammation, IL-6 is produced by monocytes and macrophages immediately after the stimulation with distinct pathogen-associated molecular patterns. In noninfectious inflammation, damage-associated molecular patterns from damaged or dying cells stimulate monocytes and macrophages to produce IL-6. The pathogenic role of IL-6 in rheumatic diseases like rheumatoid arthritis (RA) has been well established. The critical role for IL-6 in the pathogenesis of RA is provided by clinical trials, in which tocilizumab, a humanized mAb specific for IL-6R, has been shown to suppress disease activity and erosive progression in patients with RA[59]. In ReA, elevated IL-6 concentrations in the plasma and sera of the patients has been reported[60,61]. Moreover, synovial fluid concentrations of

IL-6 were higher in patients with ReA[41]. Interestingly, we found that mice TNFRp55-deficient macrophages are hyperactivated to secrete common pro-inflammatory mediators such as NO and IL-6 following stimulation with *Yersinia* antigens. The higher concentrations of IL-6 production detected in stimulated *TNFRp55*$^{-/-}$ macrophages may be associated with our previous *in vivo* results demonstrating the increased susceptibility of *TNFRp55*$^{-/-}$ mice to *Yersinia*-induced ReA[14]. Furthermore, higher concentrations of IL-6 were detected in the joints of these mice which showed a severe chronic synovitis[15]. This data suggests that over-synthesis of IL-6 may be related to the development of ReA.

TNF-α

TNF-α is a cytokine prototype of a large family of over 40, known as TNF superfamily, and TNF receptor (TNFR) proteins. TNF-α is a cytokine with pleiotropic functions produced by a large number of cells, but are monocytic lineage cells (macrophages, astroglia, microglia, Kupffer cells and alveolar macrophages) major sources. Initially, this cytokine is produced as a pro-TNF and is expressed on the cell surface. Subsequently it is cleaved by the action of a metalloproteinase (TACE) and released into the extracellular medium as a soluble protein[62]. Often, TNF-α is not detected in high concentrations in serum or tissues, but increases intensively on various inflammatory and infectious conditions. Two receptors, TNF-R1 (TNF receptor type 1; CD120a; p55/60) and TNF-R2 (TNF receptor type 2; CD120b; p75/80) bind to membrane-integrated TNF (memTNF) as well as soluble TNF-α (sTNF-α). In the vast majority of cells, TNF-R1 appears to be the key mediator of TNF-α signaling, whereas in the lymphoid system, TNF-R2 seems to play a major role. Low TNF-α secretion by blood mononuclear cells may be related to ReA development since TNF-α deficiency may interfere with eradication of bacterial infection in its early stages[34,63-66]. However, other studies suggest that TNF-α could have a pathogenic role during the chronic stage of ReA in line with the role of this cytokine in RA. In this regard, some studies have revealed significant increase of TNF-α production in chronic ReA compared with acute ReA[66]. These data support the possibility that anti-TNF-α treatment in ReA during the chronic phase of the disease could be beneficial. However, considering that TNF-α may be required for the elimination of ReA-associated bacteria, anti-TNF-α biologics might favor bacteria growth. Results obtained in our laboratory showed that TNFRp55 deficiency favors the development of ReA after infection with *Y. enterocolitica*[14]. These data support the idea that the relative lack of TNF-α may play a protective role in ReA at acute phase of disease. On the other hand, we have demonstrated an *in vivo* regulatory role for TNFRp55 signaling in fine-tuning of Th17 and Th1 programs during bacterial-induced ReA through modulation of the common p40 subunit of IL-23 and IL-12[15]. This evidence suggests that TNF-α might have a dual role in ReA, playing a protective role first and during the initial stage. However, during the chronic stage of the disease, TNF-α would act as a pro-inflammatory cytokine.

IFN-γ

IFN-γ is produced mainly by natural killer (NK) cells and a particular subset of T cells, namely Th1 cells[67]. As previously mentioned, IL-12 is the main stimulator of IFN-γ production[47,50]. Thus, IL-12 and IFN-γ coordinate the link between pathogen recognition by innate immune cells and the induction of specific immunity by mediating a positive feedback loop to amplify the Th1 response. The functional IFN-receptor (IFN-R) consists of 2 ligand-binding IFNGR1 chains and 2 signal-transducing IFNGR2 chains[68]. Mice deficient in IFN-γ or its receptor are susceptible to an array of intracellular pathogens[69-71]. It was thought that Th1 cells cause damage in the joints mainly through IFN-γ driven inflammatory mechanisms. However, similar to TNF-α, conflicting data have been reported about the role of IFN-γ in ReA. As previously mentioned, some authors have reported an aberrant lower production of IFN-γ in patients with ReA[28-34,52]. In contrast, in patients with *C. trachomatis*-induced ReA, the synovial fluid concentrations of IFN-γ were significantly higher than in OA patients but no significant differences were found between ReA and RA patients[45]. Similar results were reported by Singh *et al*[41]. Other studies have shown that the percentages of IFN-γ positive CD3$^+$ cells were significantly higher in peripheral blood and synovial fluid of chronic ReA patients[66]. These data support the idea that, as with TNF-α, IFN-γ may play a significant protective role in ReA in the acute phase of disease. However, in the chronic phase, this cytokine, as in RA, could play a pathogenic role in ReA.

IL-10

IL-10 is an anti-inflammatory cytokine with a major role in preventing inflammatory and autoimmune pathologies[72]. Based on a large body of evidence, T cells are thought to be the main source of IL-10 *in vivo*. Regulatory T (Treg) subsets are also a key source of IL-10 *in vivo* and play a central role in mediating the inflammation control. However, it is now accepted that IL-10 is expressed by subsets of all CD4$^+$ T helper populations, including Th1, Th2 and Th17[73]. Nevertheless, this cytokine is also expressed by B cells and cells of the innate immune system (DCs, stimulated macrophages, mast cells, NK cells, eosinophils and neutrophils)[74]. This cytokine binds to IL-10 receptor (IL-10R), which consists of two subunits. They are members of the interferon receptor family and belong to JAK/STAT3 class of receptors[74]. Extensive studies have demonstrated that IL-10 inhibits the production of pro-inflammatory cytokines and chemokines in activated monocytes/macrophages and inhibits proliferation of CD4$^+$ T cells[75]. However, the role of IL-10 in ReA is less clear. Appel *et al*[32] reported that the amount of IL-10 and TGF-β secreting cells was higher in ReA than in RA patients. This result was accompanied by a lower level of TNF-α secretion in ReA patients. Interest-

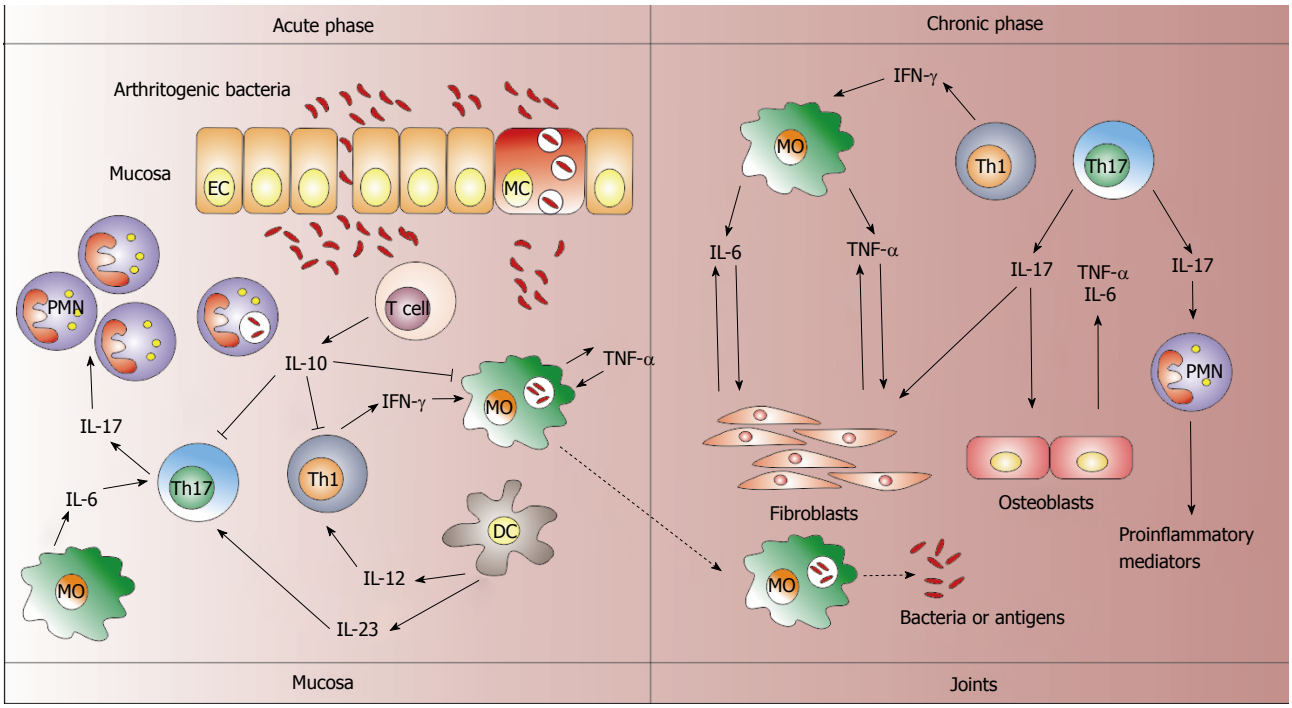


Figure 1 The role of cytokines in reactive arthritis depending on the state of disease. Arthritogenic bacteria enter through the gastrointestinal or genitourinary mucosa using different strategies (M cells; epithelial cells) and induce an inflammatory response. During the acute stage, interleukin (IL)-12 and IL-23 plus IL-6 promote the development of Th1 and Th17 cells, respectively. These cells are a major source of interferon (IFN)-γ and IL-17, favoring the bacterial clearance. The IFN-γ activates macrophages to kill phagocyted bacteria and secrete tumor necrosis factor (TNF)-α. IL-17 induces the migration of polymorphonuclear cells to the site of infection. However, this effect could be disrupted by the action of regulatory T cells producing IL-10. This regulatory event contributes to the bacterial persistence in the mucosa. Then, the bacteria could reach the joint transported by macrophages. In chronic stages, IL-6, TNF-α, IFN-γ and IL-17 exert pro-inflammatory roles in the joint. These cytokines stimulate articular cells (*e.g.*, fibroblasts, osteoblast) and immune cells to produce more cytokines and pro-inflammatory mediators that contribute to chronic articular inflammation. These effects may be enhanced by the presence of bacteria or bacterial antigens in the joint. EC: Epithelial cell; MC: M cell; MO: Macrophage; PMN: Polymorphonuclear cell; DC: Dendritic cell.

ingly, all ReA patients had a disease course of less than 6 mo. These authors suggest that this cytokine milieu might contribute to the lack of elimination of the triggering agent. Similar results were reported by Yin *et al*[28]. These authors found that synovial fluid mononuclear cells secreted low amounts of IFN-γ and TNF-α, but high amounts of IL-10 upon stimulation with specific bacteria, which was responsible for the suppression of IFN-γ and TNF-α[28]. There is also evidence indicating association of the IL-10 promoter region with the development of ReA. This raises the possibility that high levels of IL-10 in the joints of patients with ReA may be genetically determined, making these individuals more prone to the persistence of arthritogenic bacteria[76].

Despite these clinical findings suggesting a pathogenic role of IL-10 in human ReA, IL-10 depletion and IL-10 treatment in other types of arthritis models have demonstrated the anti-inflammatory properties of IL-10 in arthritis[77-80]. Results obtained in our laboratory showed that the number of Treg cells as well as the *FoxP3* mRNA expression and IL-10 levels were significantly decreased in joint regional lymph nodes of *TNFRβ55*$^{-/-}$ mice at the arthritis onset[81]. These results would indicate that IL-10 plays a protective role during the acute phase of arthritis. However, the clinical evidence suggests that high levels of IL-10 could promote bacterial persistence, favoring the development of ReA.

TREATMENT BASED ON BIOLOGICAL AGENTS

IL-6 antagonists

Published data on the effects of IL-6 blockade in patients with SpA are very scarce. Thus, in 1996 a report describes a patient with ReA who received a murine anti-IL-6 antibody[82] and, in 2009, tocilizumab was reported to be successful in another patient with ReA[83]. Only two injections of tocilizumab led to complete clinical remission from symptoms caused by ReA[83]. Recently, Kwan *et al*[84] reported successful results of tocilizumab in the treatment of a case of ReA precipitated by intravesical bacillus Galmette-Guèrin (BCG) which did not respond completely to disease modifying antirheumatic drugs (DMARDs). As previously mentioned, IL-6 is one of the cytokines that favor the differentiation of naïve T cells into Th17 cells[39]. Therefore, it is possible that the inhibitory action of tocilizumab is exerted indirectly interfering with the differentiation of Th17 cells. These data indicate that IL-6 may play a pivotal role directly or indirectly in the pathogenesis of ReA and tocilizumab treatment can be an option for an alternative treatment.

TNF-antagonists

The pathogenic role during the critical stage of the disease supports the idea that TNF-α blocking agents could be an effective treatment for patients with ReA who develop severe arthritis that does not respond to conventional lines of treatment. Thus, Kaipiainen-Seppönen *et al*[85] reported two cases of ReA post *Y. enterocolitica* treated early with infliximab (an anti-TNF-α antibody). One patient that received this treatment within 2 mo after the disease onset exhibited an improvement after the third infusion. The second patient that was treated after one month of evolution showed an immediate clinical improvement with almost complete regression after 15 d[85]. Recently, Thomas-Pohl *et al*[86] obtained the same result in one patient with ReA triggered by a gastrointestinal infection. Similar results were reported by Edrees in a patient with a severe case of *C. trachomatis*-related ReA that was successfully treated with etanercept (a fusion protein of TNFRp75)[87]. Thus, anti-TNF-α therapy has proved efficacious in some cases. However, sufficient data are lacking and theoretical concerns with their use remain. Large controlled trials are needed to evaluate the role of TNF-α blocking agents in ReA.

CONCLUSION

The network of cytokines is complex with feedback regulatory circuits that make it difficult to elucidate the role of a particular cytokine in ReA. In addition, the clinical reports of cytokine levels in patients with ReA have included patients in different stages of the disease or they are not large enough to support the role of different cytokines in ReA development. However, the current evidence in patients with anti-cytokine treatments suggests that IL-6 and TNF-α may play central roles in ReA pathogenesis. Furthermore, the IL-17/23 axis should be considered in the picture of ReA development, although further investigations are necessary for these cytokines. According to the presented evidence in this review, Figure 1 shows the different functions of the cytokines in ReA depending on the disease phases. Moreover, animal models may contribute to provide insight into the immunopathogenic mechanisms mediated by a particular cytokine in ReA and to support anti-cytokine treatments.

REFERENCES

1 **Townes JM**. Reactive arthritis after enteric infections in the United States: the problem of definition. *Clin Infect Dis* 2010; **50**: 247-254 [PMID: 20025528 DOI: 10.1086/649540]
2 **Carter JD**, Hudson AP. Reactive arthritis: clinical aspects and medical management. *Rheum Dis Clin North Am* 2009; **35**: 21-44 [PMID: 19480995 DOI: 10.1016/j.rdc.2009.03.010]
3 **Hannu T**. Reactive arthritis. *Best Pract Res Clin Rheumatol* 2011; **25**: 347-357 [PMID: 22100285 DOI: 10.1016/j.berh.2011.01.018]
4 **Leirisalo-Repo M**. Reactive arthritis. *Scand J Rheumatol* 2005; **34**: 251-259 [PMID: 16195157 DOI: 10.1080/03009740500202540]
5 **Braun J**, Kingsley G, van der Heijde D, Sieper J. On the difficulties of establishing a consensus on the definition of and diagnostic investigations for reactive arthritis. Results and discussion of a questionnaire prepared for the 4th International Workshop on Reactive Arthritis, Berlin, Germany, July 3-6, 1999. *J Rheumatol* 2000; **27**: 2185-2192 [PMID: 10990232]
6 **Rudwaleit M**, Richter S, Braun J, Sieper J. Low incidence of reactive arthritis in children following a salmonella outbreak. *Ann Rheum Dis* 2001; **60**: 1055-1057 [PMID: 11602478 DOI: 10.1136/ard.60.11.1055]
7 **Mattila L**, Leirisalo-Repo M, Koskimies S, Granfors K, Siitonen A. Reactive arthritis following an outbreak of Salmonella infection in Finland. *Br J Rheumatol* 1994; **33**: 1136-1141 [PMID: 8000742 DOI: 10.1093/rheumatology/33.12.1136]
8 **Mattila L**, Leirisalo-Repo M, Pelkonen P, Koskimies S, Granfors K, Siitonen A. Reactive arthritis following an outbreak of Salmonella Bovismorbificans infection. *J Infect* 1998; **36**: 289-295 [PMID: 9661939 DOI: 10.1016/S0163-4453(98)94243-8]
9 **Colmegna I**, Cuchacovich R, Espinoza LR. HLA-B27-associated reactive arthritis: pathogenetic and clinical considerations. *Clin Microbiol Rev* 2004; **17**: 348-369 [PMID: 15084505 DOI: 10.1128/CMR.17.2.348-369.2004]
10 **Hill JL**, Yu DT. Development of an experimental animal model for reactive arthritis induced by Yersinia enterocolitica infection. *Infect Immun* 1987; **55**: 721-726 [PMID: 3493220]
11 **Yong Z**, Hill JL, Hirofuji T, Mander M, Yu DT. An experimental mouse model of Yersinia-induced reactive arthritis. *Microb Pathog* 1988; **4**: 305-310 [PMID: 3200165 DOI: 10.1016/0882-4010(88)90091-5]
12 **Merilahti-Palo R**, Gripenberg-Lerche C, Söderström KO, Toivanen P. Long term follow up of SHR rats with experimental yersinia associated arthritis. *Ann Rheum Dis* 1992; **51**: 91-96 [PMID: 1540047]
13 **de los Toyos JR**, Vázquez J, Sampedro A, Hardisson C. Yersinia enterocolitica serotype O: 3 is arthritogenic for mice. *Microb Pathog* 1990; **8**: 363-370 [PMID: 2215184 DOI: 10.1016/0882-4010(90)90095-8]
14 **Di Genaro MS**, Cargnelutti DE, Eliçabe JR, Lacoste MG, Valdez S, Gómez N, de Guzmán AM. Role of TNFRp55 in Yersinia enterocolitica O: 3-induced arthritis: triggering bacterial antigens and articular immune response. *Rheumatology* (Oxford) 2007; **46**: 590-596 [PMID: 17043042 DOI: 10.1093/rheumatology/kel348]
15 **Eliçabe RJ**, Cargnelutti E, Serer MI, Stege PW, Valdez SR, Toscano MA, Rabinovich GA, Di Genaro MS. Lack of TNFR p55 results in heightened expression of IFN-γ and IL-17 during the development of reactive arthritis. *J Immunol* 2010; **185**: 4485-4495 [PMID: 20810989 DOI: 10.4049/jimmunol.0902245]
16 **Noto Llana M**, Sarnacki SH, Giacomodonato MN, Caccuri RL, Blanco GA, Cerquetti MC. Sublethal infection with Salmonella Enteritidis by the natural route induces intestinal and joint inflammation in mice. *Microbes Infect* 2009; **11**: 74-82 [PMID: 19022393 DOI: 10.1016/j.micinf.2008.10.010]
17 **Noto Llana M**, Sarnacki SH, Vázquez MV, Gartner AS, Giacomodonato MN, Cerquetti MC. Salmonella enterica induces joint inflammation and expression of interleukin-17 in draining lymph nodes early after onset of enterocolitis in mice. *Infect Immun* 2012; **80**: 2231-2239 [PMID: 22493084 DOI: 10.1128/IAI.00324-12]
18 **Rich E**, Hook EW, Alarcón GS, Moreland LW. Reactive arthritis in patients attending an urban sexually transmitted diseases clinic. *Arthritis Rheum* 1996; **39**: 1172-1177 [PMID: 8670327 DOI: 10.1002/art.1780390715]
19 **Schumacher HR**, Gérard HC, Arayssi TK, Pando JA, Branigan PJ, Saaibi DL, Hudson AP. Lower prevalence of Chlamydia pneumoniae DNA compared with Chlamydia trachomatis DNA in synovial tissue of arthritis patients. *Arthritis Rheum* 1999; **42**: 1889-1893 [PMID: 10513803]
20 **Bas S**, Griffais R, Kvien TK, Glennås A, Melby K, Vischer TL. Amplification of plasmid and chromosome Chlamydia

DNA in synovial fluid of patients with reactive arthritis and undifferentiated seronegative oligoarthropathies. *Arthritis Rheum* 1995; **38**: 1005-1013 [PMID: 7612032 DOI: 10.1002/art.1780380718]

21 **Gérard HC**, Carter JD, Hudson AP. Chlamydia trachomatis is present and metabolically active during the remitting phase in synovial tissues from patients with chronic Chlamydia-induced reactive arthritis. *Am J Med Sci* 2013; **346**: 22-25 [PMID: 23792903 DOI: 10.1097/MAJ.0b013e3182648740]

22 **Granfors K**, Jalkanen S, von Essen R, Lahesmaa-Rantala R, Isomäki O, Pekkola-Heino K, Merilahti-Palo R, Saario R, Isomäki H, Toivanen A. Yersinia antigens in synovial-fluid cells from patients with reactive arthritis. *N Engl J Med* 1989; **320**: 216-221 [PMID: 2643047 DOI: 10.1056/NEJM198901263200404]

23 **Granfors K**, Jalkanen S, Lindberg AA, Mäki-Ikola O, von Essen R, Lahesmaa-Rantala R, Isomäki H, Saario R, Arnold WJ, Toivanen A. Salmonella lipopolysaccharide in synovial cells from patients with reactive arthritis. *Lancet* 1990; **335**: 685-688 [PMID: 1690327 DOI: 10.1016/0140-6736(90)90804-E]

24 **Zhang Y**, Gripenberg-Lerche C, Söderström KO, Toivanen A, Toivanen P. Antibiotic prophylaxis and treatment of reactive arthritis. Lessons from an animal model. *Arthritis Rheum* 1996; **39**: 1238-1243 [PMID: 8670337 DOI: 10.1002/art.1780390725]

25 **Granfors K**, Merilahti-Palo R, Luukkainen R, Möttönen T, Lahesmaa R, Probst P, Märker-Hermann E, Toivanen P. Persistence of Yersinia antigens in peripheral blood cells from patients with Yersinia enterocolitica O: 3 infection with or without reactive arthritis. *Arthritis Rheum* 1998; **41**: 855-862 [PMID: 9588737]

26 **Gracey E**, Inman RD. Chlamydia-induced ReA: immune imbalances and persistent pathogens. *Nat Rev Rheumatol* 2012; **8**: 55-59 [PMID: 22105240 DOI: 10.1038/nrrheum.2011.173]

27 **Anttonen K**, Orpana A, Leirisalo-Repo M, Repo H. Aberrant TNF secretion by whole blood in healthy subjects with a history of reactive arthritis: time course in adherent and non-adherent cultures. *Ann Rheum Dis* 2006; **65**: 372-378 [PMID: 16107515 DOI: 10.1136/ard.2005.035972]

28 **Yin Z**, Braun J, Neure L, Wu P, Liu L, Eggens U, Sieper J. Crucial role of interleukin-10/interleukin-12 balance in the regulation of the type 2 T helper cytokine response in reactive arthritis. *Arthritis Rheum* 1997; **40**: 1788-1797 [PMID: 9336412 DOI: 10.1002/art.1780401010]

29 **Simon AK**, Seipelt E, Sieper J. Divergent T-cell cytokine patterns in inflammatory arthritis. *Proc Natl Acad Sci USA* 1994; **91**: 8562-8566 [PMID: 8078923 DOI: 10.1073/pnas.91.18.8562]

30 **Smeets TJ**, Dolhain RJ, Breedveld FC, Tak PP. Analysis of the cellular infiltrates and expression of cytokines in synovial tissue from patients with rheumatoid arthritis and reactive arthritis. *J Pathol* 1998; **186**: 75-81 [PMID: 9875143]

31 **Kotake S**, Schumacher HR, Arayssi TK, Gérard HC, Branigan PJ, Hudson AP, Yarboro CH, Klippel JH, Wilder RL. Gamma interferon and interleukin-10 gene expression in synovial tissues from patients with early stages of Chlamydia-associated arthritis and undifferentiated oligoarthritis and from healthy volunteers. *Infect Immun* 1999; **67**: 2682-2686 [PMID: 10225943]

32 **Appel H**, Neure L, Kuhne M, Braun J, Rudwaleit M, Sieper J. An elevated level of IL-10- and TGFbeta-secreting T cells, B cells and macrophages in the synovial membrane of patients with reactive arthritis compared to rheumatoid arthritis. *Clin Rheumatol* 2004; **23**: 435-440 [PMID: 15459815 DOI: 10.1007/s10067-004-0916-5]

33 **van Holten J**, Smeets TJ, Blankert P, Tak PP. Expression of interferon beta in synovial tissue from patients with rheumatoid arthritis: comparison with patients with osteoarthritis and reactive arthritis. *Ann Rheum Dis* 2005; **64**: 1780-1782 [PMID: 15878901 DOI: 10.1136/ard.2005.040477]

34 **Braun J**, Yin Z, Spiller I, Siegert S, Rudwaleit M, Liu L, Radbruch A, Sieper J. Low secretion of tumor necrosis factor alpha, but no other Th1 or Th2 cytokines, by peripheral blood mononuclear cells correlates with chronicity in reactive arthritis. *Arthritis Rheum* 1999; **42**: 2039-2044 [PMID: 10524674]

35 **Suzuki E**, Mellins ED, Gershwin ME, Nestle FO, Adamopoulos IE. The IL-23/IL-17 axis in psoriatic arthritis. *Autoimmun Rev* 2014; **13**: 496-502 [PMID: 24424175 DOI: 10.1016/j.autrev.2014.01.050]

36 **Hickman-Brecks CL**, Racz JL, Meyer DM, LaBranche TP, Allen PM. Th17 cells can provide B cell help in autoantibody induced arthritis. *J Autoimmun* 2011; **36**: 65-75 [PMID: 21075597 DOI: 10.1016/j.jaut.2010.10.007]

37 **Koenders MI**, Lubberts E, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, Di Padova FE, Boots AM, Gram H, Joosten LA, van den Berg WB. Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. *Am J Pathol* 2005; **167**: 141-149 [PMID: 15972960 DOI: 10.1016/S0002-9440(10)62961-6]

38 **Nakae S**, Saijo S, Horai R, Sudo K, Mori S, Iwakura Y. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. *Proc Natl Acad Sci USA* 2003; **100**: 5986-5990 [PMID: 12721360 DOI: 10.1073/pnas.1035999100]

39 **Bedoya SK**, Lam B, Lau K, Larkin J. Th17 cells in immunity and autoimmunity. *Clin Dev Immunol* 2013; **2013**: 986789 [PMID: 24454481 DOI: 10.1155/2013/986789]

40 **Singh AK**, Misra R, Aggarwal A. Th-17 associated cytokines in patients with reactive arthritis/undifferentiated spondyloarthropathy. *Clin Rheumatol* 2011; **30**: 771-776 [PMID: 21181220 DOI: 10.1007/s10067-010-1646-5]

41 **Singh R**, Aggarwal A, Misra R. Th1/Th17 cytokine profiles in patients with reactive arthritis/undifferentiated spondyloarthropathy. *J Rheumatol* 2007; **34**: 2285-2290 [PMID: 17937463 DOI: 10.3899/jrheum.110849]

42 **Jandus C**, Bioley G, Rivals JP, Dudler J, Speiser D, Romero P. Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides. *Arthritis Rheum* 2008; **58**: 2307-2317 [PMID: 18668556 DOI: 10.1002/art.23655]

43 **Wendling D**, Cedoz JP, Racadot E, Dumoulin G. Serum IL-17, BMP-7, and bone turnover markers in patients with ankylosing spondylitis. *Joint Bone Spine* 2007; **74**: 304-305 [PMID: 17369068 DOI: 10.1016/j.jbspin.2006.11.005]

44 **Shen H**, Goodall JC, Gaston JS. Frequency and phenotype of T helper 17 cells in peripheral blood and synovial fluid of patients with reactive arthritis. *J Rheumatol* 2010; **37**: 2096-2099 [PMID: 20634245 DOI: 10.3899/jrheum.100146]

45 **Bas S**, Neff L, Viatte S, Vuillet M, Spenato U, Guerne PA, Michel M, Tiercy JM, Butrimiene I, Gabay C. Relationship between gamma-interferon and interleukin-17 in Chlamydia trachomatis reactive arthritis. *Clin Exp Rheumatol* 2009; **27**: 885-886 [PMID: 19917178]

46 **Noto Llana M**, Sarnacki SH, Aya Castañeda Mdel R, Bernal MI, Giacomodonato MN, Cerquetti MC. Consumption of Lactobacillus casei fermented milk prevents Salmonella reactive arthritis by modulating IL-23/IL-17 expression. *PLoS One* 2013; **8**: e82588 [PMID: 24340048 DOI: 10.1371/journal.pone.0082588]

47 **Trinchieri G**. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003; **3**: 133-146 [PMID: 12563297 DOI: 10.1038/nri1001]

48 **Oppmann B**, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churakova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams JS, Moore KW, Rennick D, de Waal-Malefyt R, Hannum C, Bazan JF, Kastelein RA. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 2000; **13**: 715-725 [PMID: 11114383 DOI: 10.1016/

S1074-7613(00)00070-4]
49 **Parham C**, Chirica M, Timans J, Vaisberg E, Travis M, Cheung J, Pflanz S, Zhang R, Singh KP, Vega F, To W, Wagner J, O'Farrell AM, McClanahan T, Zurawski S, Hannum C, Gorman D, Rennick DM, Kastelein RA, de Waal Malefyt R, Moore KW. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. *J Immunol* 2002; **168**: 5699-5708 [PMID: 12023369]
50 **Chan SH**, Perussia B, Gupta JW, Kobayashi M, Pospísil M, Young HA, Wolf SF, Young D, Clark SC, Trinchieri G. Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J Exp Med* 1991; **173**: 869-879 [PMID: 1672545]
51 **Haraguchi S**, Day NK, Nelson RP, Emmanuel P, Duplantier JE, Christodoulou CS, Good RA. Interleukin 12 deficiency associated with recurrent infections. *Proc Natl Acad Sci USA* 1998; **95**: 13125-13129 [PMID: 9789052 DOI: 10.1073/pnas.95.22.13125]
52 **Bas S**, Kvien TK, Buchs N, Fulpius T, Gabay C. Lower level of synovial fluid interferon-gamma in HLA-B27-positive than in HLA-B27-negative patients with Chlamydia trachomatis reactive arthritis. *Rheumatology* (Oxford) 2003; **42**: 461-467 [PMID: 12626797 DOI: 10.1093/rheumatology/keg163]
53 **Välimäki E**, Aittomäki S, Karenko L, Kantonen J, Pettersson T, Turunen U, Matikainen S, Leirisalo-Repo M, Repo H. Normal inflammasome activation and low production of IL-23 by monocyte-derived macrophages from subjects with a history of reactive arthritis. *Scand J Rheumatol* 2013; **42**: 294-298 [PMID: 23425136 DOI: 10.3109/03009742.2012.754940]
54 **Wendling D**, Cedoz JP, Racadot E. Serum and synovial fluid levels of p40 IL12/23 in spondyloarthropathy patients. *Clin Rheumatol* 2009; **28**: 187-190 [PMID: 18827961 DOI: 10.1007/s10067-008-1011-0]
55 **Di Genaro MS**, Cargnelutti DE, Castro DO, Eliçabe RJ, Gutiérrez JV, Correa SG, de Guzmán AM. Yersinia-triggered arthritis in IL-12p40-deficient mice: relevant antigens and local expression of Toll-like receptor mRNA. *Scand J Rheumatol* 2007; **36**: 28-35 [PMID: 17454932 DOI: 10.1080/03009740600906651]
56 **Yamasaki K**, Taga T, Hirata Y, Yawata H, Kawanishi Y, Seed B, Taniguchi T, Hirano T, Kishimoto T. Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. *Science* 1988; **241**: 825-828 [PMID: 3136546 DOI: 10.1126/science.3136546]
57 **Hibi M**, Murakami M, Saito M, Hirano T, Taga T, Kishimoto T. Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell* 1990; **63**: 1149-1157 [PMID: 2261637 DOI: 10.1016/0092-8674(90)90411-7]
58 **Taga T**, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T, Kishimoto T. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* 1989; **58**: 573-581 [PMID: 2788034 DOI: 10.1016/0092-8674(89)90438-8]
59 **Smolen JS**, Beaulieu A, Rubbert-Roth A, Ramos-Remus C, Rovensky J, Alecock E, Woodworth T, Alten R. Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. *Lancet* 2008; **371**: 987-997 [PMID: 18358926 DOI: 10.1016/S0140-6736(08)60453-5]
60 **Metsärinne KP**, Nordström DC, Konttinen YT, Teppo AM, Fyhrquist FY. Plasma interleukin-6 and renin substrate in reactive arthritis, rheumatoid arthritis, and systemic lupus erythematosus. *Rheumatol Int* 1992; **12**: 93-96 [PMID: 1384103 DOI: 10.1007/BF00290261]
61 **Straub RH**, Paimela L, Peltomaa R, Schölmerich J, Leirisalo-Repo M. Inadequately low serum levels of steroid hormones in relation to interleukin-6 and tumor necrosis factor in untreated patients with early rheumatoid arthritis and reactive arthritis. *Arthritis Rheum* 2002; **46**: 654-662 [PMID: 11920401 DOI: 10.1002/art.10177]
62 **Wajant H**, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ* 2003; **10**: 45-65 [PMID: 12655295 DOI: 10.1038/sj.cdd.4401189]
63 **Westendorp RG**, Langermans JA, de Bel CE, Meinders AE, Vandenbroucke JP, van Furth R, van Dissel JT. Release of tumor necrosis factor: an innate host characteristic that may contribute to the outcome of meningococcal disease. *J Infect Dis* 1995; **171**: 1057-1060 [PMID: 7706790 DOI: 10.1093/infdis/171.4.1057]
64 **Netea MG**, van der Meer JW, van Deuren M, Kullberg BJ. Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? *Trends Immunol* 2003; **24**: 254-258 [PMID: 12738419 DOI: 10.1016/S1471-4906(03)00079-6]
65 **Saleh M**, Vaillancourt JP, Graham RK, Huyck M, Srinivasula SM, Alnemri ES, Steinberg MH, Nolan V, Baldwin CT, Hotchkiss RS, Buchman TG, Zehnbauer BA, Hayden MR, Farrer LA, Roy S, Nicholson DW. Differential modulation of endotoxin responsiveness by human caspase-12 polymorphisms. *Nature* 2004; **429**: 75-79 [PMID: 15129283 DOI: 10.1038/nature02451]
66 **Butrimiene I**, Jarmalaite S, Ranceva J, Venalis A, Jasiuleviciute L, Zvirbliene A. Different cytokine profiles in patients with chronic and acute reactive arthritis. *Rheumatology* (Oxford) 2004; **43**: 1300-1304 [PMID: 15266062 DOI: 10.1093/rheumatology/keh323]
67 **Frucht DM**, Fukao T, Bogdan C, Schindler H, O'Shea JJ, Koyasu S. IFN-gamma production by antigen-presenting cells: mechanisms emerge. *Trends Immunol* 2001; **22**: 556-560 [PMID: 11574279 DOI: 10.1016/S1471-4906(01)02005-1]
68 **Schroder K**, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 2004; **75**: 163-189 [PMID: 14525967 DOI: 10.1189/jlb.0603252]
69 **Flynn JL**, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med* 1993; **178**: 2249-2254 [PMID: 7504064 DOI: 10.1084/jem.178.6.2249]
70 **Huang S**, Hendriks W, Althage A, Hemmi S, Bluethmann H, Kamijo R, Vilcek J, Zinkernagel RM, Aguet M. Immune response in mice that lack the interferon-gamma receptor. *Science* 1993; **259**: 1742-1745 [PMID: 8456301 DOI: 10.1126/science.8456301]
71 **Harty JT**, Bevan MJ. Specific immunity to Listeria monocytogenes in the absence of IFN gamma. *Immunity* 1995; **3**: 109-117 [PMID: 7621071 DOI: 10.1016/1074-7613(95)90163-9]
72 **O'Garra A**, Barrat FJ, Castro AG, Vicari A, Hawrylowicz C. Strategies for use of IL-10 or its antagonists in human disease. *Immunol Rev* 2008; **223**: 114-131 [PMID: 18613832 DOI: 10.1111/j.1600-065X.2008.00635.x]
73 **Hedrich CM**, Bream JH. Cell type-specific regulation of IL-10 expression in inflammation and disease. *Immunol Res* 2010; **47**: 185-206 [PMID: 20087682 DOI: 10.1007/s12026-009-8150-5]
74 **Yao Y**, Simard AR, Shi FD, Hao J. IL-10-producing lymphocytes in inflammatory disease. *Int Rev Immunol* 2013; **32**: 324-336 [PMID: 23617759 DOI: 10.3109/08830185.2012.762361]
75 **Moore KW**, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001; **19**: 683-765 [PMID: 11244051 DOI: 10.1146/annurev.immunol.19.1.683]
76 **Kaluza W**, Leirisalo-Repo M, Märker-Hermann E, Westman P, Reuss E, Hug R, Mastrovic K, Stradmann-Bellinghausen B, Granfors K, Galle PR, Höhler T. IL10.G microsatellites mark promoter haplotypes associated with protection against the

development of reactive arthritis in Finnish patients. *Arthritis Rheum* 2001; **44**: 1209-1214 [PMID: 11352256]
77 **Finnegan A**, Kaplan CD, Cao Y, Eibel H, Glant TT, Zhang J. Collagen-induced arthritis is exacerbated in IL-10-deficient mice. *Arthritis Res Ther* 2003; **5**: R18-R24 [PMID: 12716449 DOI: 10.1186/ar601]
78 **Finnegan A**, Mikecz K, Tao P, Glant TT. Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. *J Immunol* 1999; **163**: 5383-5390 [PMID: 10553063]
79 **Fellowes R**, Etheridge CJ, Coade S, Cooper RG, Stewart L, Miller AD, Woo P. Amelioration of established collagen induced arthritis by systemic IL-10 gene delivery. *Gene Ther* 2000; **7**: 967-977 [PMID: 10849557]
80 **Kasama T**, Strieter RM, Lukacs NW, Lincoln PM, Burdick MD, Kunkel SL. Interleukin-10 expression and chemokine regulation during the evolution of murine type II collagen-induced arthritis. *J Clin Invest* 1995; **95**: 2868-2876 [PMID: 7769128 DOI: 10.1172/JCI117993]
81 **Cargnelutti E**, Di Genaro MS. Reactive Arthritis: from clinical features to pathogenesis. *Int J Clin Med* 2013; **4**: 20-30 [DOI: 10.4236/ijcm.2013.412A2004]
82 **Wendling D**, Racadot E, Toussirot E, Wijdenes J. Combination therapy of anti-CD4 and anti-IL6 monoclonal antibodies in a case of severe spondylarthropathy. *Br J Rheumatol* 1996; **35**: 1330 [PMID: 9010072 DOI: 10.1093/rheumatology/35.12.1330]
83 **Tanaka T**, Kuwahara Y, Shima Y, Hirano T, Kawai M, Ogawa M, Arimitsu J, Hagihara K, Narazaki M, Ogata A, Kawase I, Kishimoto T. Successful treatment of reactive arthritis with a humanized anti-interleukin-6 receptor antibody, tocilizumab. *Arthritis Rheum* 2009; **61**: 1762-1764 [PMID: 19950316 DOI: 10.1002/art.24899]
84 **Kwan K**, Bharadwaj S, Inderjeeth C. Response to treatment with tocilizumab of reactive arthritis induced by intravesical bacillus Galmette-Guérin unresponsive to DMARDs. *Int J Rheum Dis* 2012; **15**: e73-e75 [PMID: 22898231 DOI: 10.1111/j.1756-185X.2012.01735.x]
85 **Kaipiainen-Seppönen O**, Niinisalo H, Korpilähde T, Virolainen J. Treatment of reactive arthritis with infliximab. *Scand J Rheumatol* 2003; **32**: 122-124 [PMID: 12737333 DOI: 10.1080/03009740310000157]
86 **Thomas-Pohl M**, Tissot A, Banal F, Lechevalier D. Spectacular evolution of reactive arthritis after early treatment with infliximab. *Joint Bone Spine* 2012; **79**: 524 [PMID: 22542050 DOI: 10.1016/j.jbspin.2012.03.001]
87 **Edrees A**. Successful use of etanercept for the treatment of Reiter's syndrome: a case report and review of the literature. *Rheumatol Int* 2012; **32**: 1-3 [PMID: 21785961 DOI: 10.1007/s00296-011-2000-1]

P- Reviewer: Gazouli M, Konttinen Yrj, Pixley J **S- Editor**: Ji FF
L- Editor: Roemmele A **E- Editor**: Wang CH

World Journal of Immunology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5411/wji.v4.i2.88

World J Immunol 2014 July 27; 4(2): 88-97
ISSN 2219-2824 (online)

REVIEW

Update on pythiosis immunobiology and immunotherapy

Érico S Loreto, Juliana SM Tondolo, Régis A Zanette, Sydney H Alves, Janio M Santurio

Érico S Loreto, Juliana SM Tondolo, Régis A Zanette, Sydney H Alves, Janio M Santurio, Department of Microbiology and Parasitology, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil
Érico S Loreto, Juliana SM Tondolo, Régis A Zanette, Sydney H Alves, Janio M Santurio, Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil
Author contributions: Loreto ÉS, Tondolo JSM, Zanette RA, Alves SH and Santurio JM solely contributed to this paper.
Supported by The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil, No. CAPES-AUX PE-PNPD 743/2012
Correspondence to: Dr. Janio M Santurio, Department of Microbiology and Parasitology, Universidade Federal de Santa Maria, Av. Roraima nº 1000, Prédio 20, sala 4139, Santa Maria, RS 97105-900, Brazil. janio.santurio@gmail.com
Telephone: +55-55-32208906 Fax: +55-55-32208906
Received: March 27, 2014 Revised: May 6, 2014
Accepted: June 10, 2014
Published online: July 27, 2014

Abstract

Pythiosis is an invasive, ulcerative, pyogranulomatous disease caused by *Pythium insidiosum*, a fungus-like oomycete that has been reported to affect humans, horses, dogs, and other mammals mainly in tropical and subtropical areas of the world. The disease is characterized by an eosinophilic granulomatous and a Th2 immune response which in turn helps to protect the fungus from the host cells. Pythiosis can present clinically in subcutaneous, gastrointestinal, and vascular tissues or in a systemically disseminated form depending on the species and site of infection. Changes in iron metabolism and anemia are commonly observed. The diagnosis is accomplished through clinical and pathological features, laboratory characteristics of cultures, serological and molecular tests. Treatment includes radical surgery, antimicrobial drugs, immunotherapy or a combination of these treatments. Immunotherapy is a practical and non-invasive alternative for treating pythiosis which is believed to promote a switch from a Th2 to Th1 immune response, resulting in a favorable clinical response. This therapy has demonstrated cure rates above 70% and 55% in horses and humans but low cure rates in dogs and cats. Despite the curative properties of this type of immunotherapy, the antibodies that are produced do not prevent host reinfection. Thus, development of effective adjuvants and new diagnostic techniques for early disease diagnosis are of utmost importance. The aim of this review was to promote pythiosis awareness and to provide an update about the immunotherapy and immunobiology of this disease.

Key words: *Pythium insidiosum*; Treatment; Pythiosis; Immunotherapy; Pathogenesis

Core tip: Pythiosis is a life-threatening disease for which there is no gold standard chemotherapy. Immunotherapy derived from killed mycelium from *Pythium insidiosum* is a non-invasive therapy that has demonstrated cure rates above 90% when associated with the surgical removal of the lesions and early disease diagnosis.

Loreto ÉS, Tondolo JSM, Zanette RA, Alves SH, Santurio JM. Update on pythiosis immunobiology and immunotherapy. *World J Immunol* 2014; 4(2): 88-97 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i2/88.htm DOI: http://dx.doi.org/10.5411/wji.v4.i2.88

INTRODUCTION

Pythium insidiosum (*P. insidiosum,* an oomycete also known as water mold) is a filamentous microorganism that shares many characteristics with fungi (*i.e.*, it grows by polarized hyphal extension, engages in an absorptive mode of nutrition, and it can form spores for reproduction). However, *P. insidiosum* is classified in a completely different taxonomic group, namely the Stramenopiles, together with diatoms and brown algae[1]. This classifica-

tion is the first essential information for understanding pythiosis in humans and other mammals; infections have similar clinical and histopathological characteristics as those of certain mycoses. Conversely, treating pythiosis with antifungal drugs is generally inefficient because the oomycetes do not synthesize ergosterol, which is a component of the plasma membrane of true fungi and the primary target of those drugs[2]. Similarly, the immune response of animals with pythiosis presents similar features to those of fungal infections, and there are peculiarities found only in infections caused by this oomycete[3]. The challenge of treating pythiosis is characterized by the severity of the disease in mammals and by the absence of a gold standard chemotherapy. Nevertheless, immunotherapy is a practical and non-invasive alternative for treating pythiosis, and there is a favorable clinical response. In this context, the aim of this review was to promote pythiosis awareness and to provide an update about the immunotherapy and immunobiology of this disease.

THE EPIDEMIOLOGY, IMMUNOBIOLOGY AND PATHOGENESIS OF PYTHIOSIS

The proposed life cycle of *P. insidiosum* is characterized by the colonization of aquatic plants and the soil of wetlands or swampy areas, which serve as a substrate for mycelial vegetative growth and the asexual formation of mobile biflagellate zoospores that move through the water, find another host, encyst and form a new mycelium that can then start a new colonization[4-6].

In view of this biological cycle, pythiosis cases are associated with human or animal contact with areas in which zoospore-containing water accumulates (such as wetlands and lakes) and environmental temperatures range between 30 ℃ and 40 ℃. Most reports of animal pythiosis are described in horses that live in swampy areas or periodically enter ponds or lakes. These cases are distributed primarily between the peak months of the rainy season in each region. A higher human disease frequency has been observed in thalassemic patients in Thailand, where it is common for people to work in flooded rice cultivation areas[7-9].

Human and animal pythiosis cases have been described in tropical and subtropical climatic regions. Although these cases are most often diagnosed in Australia, Asia, Latin America and United States, some cases have originated from temperate areas of Japan, South Korea, Oceania and Africa. There are no reports of animal-animal or animal-human transmission[10,11].

P. insidiosum hyphae do not exert sufficient pressure to penetrate undamaged skin through mechanical means alone[12], and they must effect a decisive reduction in tissue strength by proteinase secretion or by finding prior skin damage to invade their host. Indeed, *P. insidiosum* possesses a strong tropism for mammalian injured tissue[10]. Interestingly, *P. insidiosum* has been recovered from a mosquito larva (*Culex quinquefasciatus*)[13] and hematophagous insects prefer the same anatomical areas for blood feeding in which pythiosis lesions are more prevalent in horses[9]. Given this information, future studies should investigate if insect bites could favor the penetration of zoospores into injured skin or if infected mosquitoes could directly transmit the disease.

Once inside the host, pythiosis pathogenesis involves the Splendore-Hoeppli phenomenon (reaction), which is characterized by the presence of radiating, star-like asteroid or club-shaped eosinophilic material around the infectious agent[14]. Thus, *P. insidiosum* triggers an eosinophilic granulomatous reaction similar to other fungi, such as *Basidiobolus* spp. and *Conidiobolus* spp.[15], with characteristic histopathological features depending on the species and the clinical form[16].

Mendoza *et al*[17] proposed that the antigens released by *P. insidiosum* hyphae modulate the host's immune response and may be responsible by keeping an eosinophilic granulomatous response, locking the immune system into a Th2 immune response through the continuous stimulatory production of more eosinophils and mast cells, which in turn helps to protect the fungus from the host cells and leads to a worsening condition, and if not treated properly, can lead to host death. As a consequence, the *P. insidiosum* hyphae surrounded by degranulated eosinophils would be camouflaged inside the eosinophilic micro-abscesses, preventing their full presentation to the immune system and thereby ensuring their viable presence in infected tissues. These features and the subsequent finding of elevated IgE levels in humans and horses with the disease strongly validated the concept of a Th2 modulation by this pathogen during natural infection[3,17]. High Th2 interleukin levels [interleukin (IL)-4, IL-5 and IL-10] have also been detected in human patients with pythiosis, confirming a Th2 immune response[18,19].

The eosinophil degranulation in equids and camels with pythiosis is remarkable, forming around the hyphae cores of necrotic yellow-gray and firm materials called *kunkers*, which are easily shed from lesions[4,15,20,21]. The *kunkers* range from 2 to 10 mm in diameter, have an irregular shape and sandy aspect, may be branched and invade the granulation tissue within the sinus formed along its trajectory. This pronounced degranulation is also associated with extensive tissue damage and with the tumor-like appearance of lesions that can reach over 50 cm in diameter[22]. Horses are the mammals that are most affected by pythiosis, with no predisposition according to their age, race or sex. However, although young animals are also susceptible, the disease is rarely observed in animals under one year of age, and the bodily lesions are predominant in dark pigmented areas[9].

The lesions are subcutaneous and present primarily in the distal extremities, the ventral portion of the thoracoabdominal wall and face, which represent anatomical structures that remain in contact with contaminated water containing *P. insidiosum* zoospores[7,23,24]. The *kunkers* are considered to be pathognomonic of pythiosis in equids, and they have also been described in camels with vulvar pythiosis[21] and in a case of equine conidiobolo-

mycosis[25]. The disease was also described in cattle[26-29], cats[27,30], dogs[16,17,27,31-39], sheep[40,41] and occasionally in animals kept in captivity in zoos[42,43] and birds[44].

P. insidiosum can cause superficial infections in humans, namely keratitis with corneal involvement[45-47]; cutaneous and subcutaneous infections[48]; orbital pythiosis and bone involvement[49,50]; and systemic infections, namely arteritis of the lower limbs and/or dissemination[8,19,51]. Although pythiosis can affect apparently healthy individuals[48,50,51], most cases are reported in patients with thalassemia and other hematological diseases[8]. The same authors have argued that iron overload, which is a marked characteristic of patients with thalassemia, could increase host susceptibility to pythiosis by promoting the infectivity of the pathogen or by impairing host immunity.

In fact, both iron overload and deficiency can weaken the immune system[52-54]. Additionally, many microorganisms are known to be avid for iron during infection[53,55] and changes in iron metabolism may increase host susceptibility to infection by *P. insidiosum*. Krajaejun *et al*[56] described that *P. insidiosum* expresses a gene encoding a ferrochelatase and Krajaejun *et al*[57] reported, through the transcriptome analysis of this species, an extensive repertoire of proteins that may be involved as virulence factors during infection. Although the role of iron in pythiosis has not been fully explained, the disease is more frequently found in human patients with thalassemia and with other hemolytic anemias[18,19,58]. Anemia as a consequence of the disease has already been described in horses[59-65], dogs[33,34,37,39,66], cats[39,67], camels[21,68] and in a jaguar[42] and Bengal tiger[69]. Santos *et al*[9] also argue that the iron deficiency is common in lactating foals (< 1-year-old and, which are less susceptible to pythiosis) because of the low iron levels in the milk. In contrast, iron deficiency is uncommon in adult horses, and they may have increased levels of circulating iron, especially in the Brazilian Pantanal, which contains high levels of this mineral in the soil, plants and water[70], and where it is observed a high incidence of pythiosis.

Loreto *et al*[71] reported an increase in the unsaturated iron binding capacity (UIBC) in rabbits experimentally infected with *P. insidiosum*, suggesting that there was an increase in the transferrin concentration and/or an increase in the number of transferrin iron receptors, which is compatible with a physiological decrease in the iron availability. Similar results were observed by Zanette *et al*[72], who noted that rabbits experimentally infected with pythiosis presented decreased serum iron levels, increased transferrin levels with low saturations (increased UIBC) and markedly decreased levels of stainable iron in the hepatocytes, which suggests an affinity for iron by *P. insidiosum*.

DIAGNOSIS AND HUMORAL RESPONSE

A classical pythiosis diagnosis is accomplished through clinical and pathological features, in addition to cultural, morphological and reproductive characteristics *in vitro*. A differential diagnosis includes habronemiasis, neoplasms, exuberant granulation tissue, and fungal or bacterial granulomas[73,74]. Microscopic evaluations using 10% KOH (direct examination) can reveal *P. insidiosum* hyaline hyphae and eventually septate-morphology, depending on the clinical material evaluated. This material can easily be confused with filamentous fungi, particularly those of the orders Entomoftorales and Mucorales[11]. A culture from *kunkers* or biopsies can usually be performed on V8 agar, corn meal agar and Sabouraud dextrose agar.

Hyphal growth can be observed after 24 h of incubation at 37 ℃ when submerged in culture medium, and it exhibits a hyaline or white color[75]. Because *P. insidiosum* does not produce reproductive structures in traditional culture media, the induction of zoosporogenesis (asexual zoospore formation) can be obtained by cultivating *P. insidiosum* in sterile blades of grass that are then transferred to a mineral solution[76]. However, the correct identification of this species should be confirmed by molecular methods[77-81].

The production of anti-*P. insidiosum* antibodies was one of the first immunological features described for pythiosis, and these antibodies were easily detected by immunodiffusion and complement fixation tests with antigens that were extracted from the pathogen[82,83]. Studies then confirmed that humans and animals suffering from pythiosis exhibited a humoral immune response upon host-pathogen interaction[3,36,77,84-86], but this response was not sufficient to clear the infection[19,50,87,88]. However, the serological tests developed for detecting antibodies, such as agar gel immunodiffusion, enzyme-linked immunosorbent assay (ELISA), Western blot, latex agglutination and immunochromatographic tests[77-80,89-93], are highly useful for the early diagnosis of pythiosis. In equine pythiosis cases in which the animal is far from reference laboratories, sending serum for ELISA and collecting *kunkers* and tissues for microbiological culture and histopathological analysis are among the primary forms of diagnosis. An early pythiosis diagnosis can also be performed through immunohistochemical[16,94] and molecular methods[80,95].

TREATMENT

Antimicrobial and surgical treatment

Because primary antifungal drugs act directly or indirectly on ergosterol and *Pythium* spp. are unable to synthesize any sterols, it is understandable that pythiosis cases do not respond satisfactorily to antifungal treatments. However, contradictory results have been reported in the use of antifungal agents to treat pythiosis[8,10,49,96].

P. insidiosum isolates have varying *in vitro* susceptibility to antifungal compounds[97,98]. Reviews of antifungal drug associations show that *in vitro* synergism occurs in AmB + terbinafine[99], terbinafine + azole antifungals and terbinafine + caspofungin associations[100]. Additionally, some antibacterial drugs that act as protein synthesis inhibitors (macrolides, tetracyclines and glycylcycline) have been shown to inhibit the *in vitro* growth of *P. insidiosum*[101,102]; nonetheless, experimental *in vivo* tests have not been con-

ducted to demonstrate the clinical effectiveness of these antibiotics.

Successes and failures of pythiosis treatment cases have been reported with combinations of antifungal therapies. The surgical removal of the lesion, the amputation of the affected limb or the enucleation of the affected eye represents the last resort in human pythiosis treatment. However, recurrence rates of 40% have been observed, which illustrates the difficulty of controlling this disease[11]. The implementation of surgical treatment with antifungal drugs or potassium iodide was described in cases of therapeutic healing[73].

Surgically removing all affected tissue is the traditional and most commonly used method for equine pythiosis treatment. The surgery yields good results for small and superficial lesions. However, removing the lesion with a safety margin to avoid recurrences is often hampered by the anatomical regions that are typically involved (distal extremities and the ventral portion of the thoracoabdominal wall)[73].

Immunotherapy

Although the antigens used in vaccine preparation (usually from the infectious agent itself) are intended to trigger a protective response in the host immune system (antibody production), the aim of immunotherapy (antibodies or antigens from the infectious agent) is the objective modification of the host immune response to mount an effective response against a disease that is already present. Despite the fact that a protective vaccine against pythiosis does not currently exist, the immunotherapy developed from protein extracts of *P. insidiosum* cultures is a noninvasive alternative for treating this disease in humans and animals.

Immunotherapy was discovered by serendipity when investigators were working on a skin test for pythiosis in horses, and they found that almost half the animals were cured upon inoculation with *P. insidiosum* immunogen[88,103,104]. The first investigator to use a culture-derived antigen for a skin test was Witkamp[83], but he did not report cure rates in his experiments. Miller[103] was the first researcher to report the use of *P. insidiosum* antigens (sonicated hyphae) with therapeutic potential when injected into horses (n = 30), resulting in 53% healing in the animals with pythiosis (Table 1). During the following year, the same author observed an immunotherapeutic efficiency ratio of 75% when associated with surgical removal[105]. Subsequent studies showed that lesions presenting with more than two months of progress in cases of chronic pythiosis had cure rates of approximately 20%-40% with immunotherapy, and cure rates of 100% were obtained when the lesions had less than 20 d of evolution[17,88,104].

In addition to the lesion evolution time, the manner by which the *P. insidiosum* mycelium is broken to obtain the antigens is also associated with immunotherapy efficacy. In this context, modifications to the original technique as described by Miller[103] have been developed with the aim of increasing the effectiveness and safety of immunotherapy.

Mendoza *et al*[104] tested two immunotherapies by using the cell mass or a concentrated soluble antigen as an antigen, and they observed efficacies of 60% and 70%, respectively, when treating 71 horses. Mendoza *et al*[17] reported that immunotherapy derived from the soluble antigen and sonicated hyphae of *P. insidiosum* cured 72% of the horses (n = 18) with pythiosis.

Santurio *et al*[106] compared the immunotherapy obtained from sonication, maceration (or liquidification) or the combination of these two techniques in experimental pythiosis cases in rabbits and observed that the macerated immunotherapy had a higher efficiency, with a reduction of 71.8% in the lesion sizes and the clinical cure of two rabbits (n = 5). This macerated immunotherapy was lyophilized, and it was valid for more than one year without refrigeration[106]. This treatment exhibited a cure rate of 50% to 83% (n = 19)[107], or 75% (n = 8)[7] and 90% when combined with surgical excision (n = 11)[24] in horses in the Brazilian Pantanal. The best results are typically observed when the disease is in its early stages.

Despite the good immunotherapy performance in equines, immunotherapy in cats and dogs has been disappointing[17,27,31]. One explanation for this failure might be that most dogs and cats with pythiosis are diagnosed several months after the initial onset of infection, resulting in animals with weakened immune systems that respond poorly to immunotherapy[3]. However, the healing of a dog was demonstrated by the combination of immunotherapy and antifungal therapy[108].

The immunotherapy treatment period (no. of doses) is related to the size, location, time of lesion development, and individual patient response. Santos *et al*[24] reported that a horse with 90 d of disease evolution required five months of treatment (eight doses) for complete lesion healing, and they noted that the slowness in the immunotherapy response cannot be interpreted as refractory and in turn end in the premature withdrawal of treatment. Conversely, only two to three doses promoted the effective healing of four horses bearing lesions with seven and 45 d of development.

Field tests with macerated immunotherapy have demonstrated that the efficacy of this treatment is directly associated with early diagnosis. The borderline between a clinical cure and an unsatisfactory response or even nonresponsive cases seems to be 60 d from the appearance of lesions in horses[3]. The treatment consists of subcutaneous applications at 14-d intervals until the complete healing of the granulomatous ulcerative tissue. A mild reaction at the injection site is often observed, and in most cases, it subsides in a few weeks. The number of doses is variable, and some animals respond better to weekly applications. In fact, the only disadvantage of this treatment is the production of protective IgG classes, which impairs serodiagnostic tests such as ELISA and immunochromatography. In this context, blood collection for serological diagnosis of pythiosis should be performed

Table 1 Review of animal pythiosis cases reported in the literature when treated with immunotherapy

Species/*n*	Lesions	Adjunctive therapy	Immunotherapy type[3], doses	Outcome	Ref.
Horses					
40	Various[4]	No or surgery	UF, 3[1] doses at 7-d intervals	C (53%), I (33%)	[103]
5	Limbs	ATM, surgery	UF, 3 doses at 7-d intervals	C (20%), 60(E), 20 (D)	[60]
5	Various[4]	No	SA, 2 doses at 15-d intervals	C (60%)	[88]
1	Limb, bones	No	SA, 2 doses at 7-d intervals	E	[109]
71	Various[4]	Nr	FH or SA, 1 or 2 doses at 7-d intervals	C (66%)	[104]
1	Limb, bones	Surgery, ATM	Nr, 3 doses postsurgical	D	[110]
2	Abdomen	Surgery, ATM	SH, 3 doses at 7-d intervals	C (50%), E (50%)	[111]
19	Various[4]	No	LMH, 3 to 9 doses at 14-d intervals	C (50%-83%)	[107]
18	Various[4]	Surgery, ATM	SA + SH, 2[1] doses at 15-d intervals	C (72%)	[17]
1	Limbs, sub-maxillary	Surgery	Nr	E	[23]
1	Limb	ATM	LMH, 7 doses at 14-d intervals	D	[112]
1	Hind pastern, fetlock	ATB	SA, 3 doses at 1, 7 and 21 d	E	[113]
1	Face	Surgery, ATM	LMH, 5 doses at 14-d intervals	E	[114]
1	Face	ATM	LMH, 5 doses at 14-d intervals	C	[64]
1[2]	Limb, abdomen	No	LMH, 4-5 doses at 14-d intervals	C	[115]
11	Limbs, abdomen	No or surgery	LMH, 2-5 doses at 14-d intervals	C (70%-90%)	[24]
8	Limbs, abdomen	No or surgery	LMH, Nr	C (75%)	[7]
47	Various[4]	No or surgery	LMH, Nr	C (79%-84%)	[9]
Dogs					
1	Cutaneous	AMB, surgery	UF, 1 dose	C	[38]
6	Cutaneous, intestinal	ATM, surgery	SA + SH, 2[1] doses at 15-d intervals	C (33%)	[17]
2	Cutaneous	Itraconazole	SA, 1 or 2 doses at 7-d intervals	E	[31]
1	Cutaneous	No	SA, 2 doses at 14-d intervals	C	[35]
1	Gastrointestinal	ATF, surgery	Nr, 3 doses at 1, 7 and 21 d	C	[32]
1	Gastrointestinal	ATF	SA, 6 doses at 15-d intervals	C	[108]
Camels					
1	Face, stomach	Surgery, ATM	SA + SH, 2[1] doses at 14-d intervals	D	[68]
2	Vulvar	Surgery, ATM	SA, 3 doses at 1, 10, 17 d	C (50%)	[21]
Sheep					
6	Oronasal	No	LMH, 1-5 doses at 14-d intervals	C (16.7%)	[41]

[1]At least; [2]Same animal, cured twice with immunotherapy with reinfection within an interval of two years; [3]Manufacturing process for immunotherapy; [4]Not reported individually (subcutaneous). Nr: Not reported; AMB: Amphotericin B; UF: Ultrasonication of hyphae; SA: Soluble antigens; SH: Sonicated hyphae; FH: Fragmented hyphae; LMH: Lyophilized macerated hyphae; C: Cured; I: Clinically improved; D: Died; E: Euthanized; ATM: Antimicrobials; ATB: Antibacterials; ATF: Antifungals.

Table 2 Review of human pythiosis cases reported in the literature when treated with immunotherapy

n	Lesions	Adjunctive therapy	Immunotherapy type[2], doses	Outcome	Ref.
1	Vascular	ATM, surgery	SA, 2 doses at 14-d intervals	C	[19]
8	Vascular	Surgery/amputation, ATF	SA, 2[1] doses at 14-d intervals	C (50%)	[18]
1	Vascular	Above-knee amputation	SA, Nr	C	[116]
1	Vascular	ATM, limb amputation	SA, Nr	D	[117]
1	Ocular	ATM, enucleation	Nr	D	[118]
1	Vascular	Above-knee amputation	Nr	C	[119]
1	Vascular	ATM, above-knee amputations	Nr	C	[120]
1	Vascular	ATM, above-the-knee-amputation	Nr, 4 doses at 7-d intervals	C	[95]
1	Vascular/disseminated	ATM	SA, 2 doses at 7-d intervals	D	[121]
3	Ocular	ATM, surgery	Nr, 3 doses	C (66%)	[122]

[1]At least; [2]Manufacturing process for immunotherapy. Nr: Not reported; SA: Soluble antigens; C: Cured; D: Died; ATM: Antimicrobials; ATF: Antifungals.

before the application of immunotherapy, thus preventing false-positive results.

Because of the higher incidence of pythiosis in horses, most data on the efficacy of immunotherapy are described in this animal species[7,9,17,23,24,60,64,88,103,104,107,109-115]. However, there are also descriptions of its use in dogs[17,31,32,35,38,108], camels[21,68] and sheep[41] (Table 1). Human immunotherapy was described for both successful and failed treatments in association with surgical procedures and the use of various antimicrobials[18,19,95,116-122] (Table 2). These studies suggest that the injection of *P. insidiosum* immunogens in the form of immunotherapy make antigens available to the host immune system that are not produced during active infection, stimulating a healing response and the formation of immune responses with the presence of mononuclear cells and the disappearance of the eosinophilic reaction around the hyphae.

The proposed mechanism for immunotherapy success is based on a change in the type of cellular response. The immune response observed during pythiosis involves eosinophilic inflammation and the expression of T helper lymphocyte type 2 (Th2) with the release of interleukins 4 and 5 and the mobilization of eosinophils and mast cells. However, the expression of T helper lymphocyte type 1 (Th1) occurs after the immunotherapeutic treatment with the release of interleukin 2 and INF-γ and the mobilization of T lymphocytes and macrophages, which destroy the *P. insidiosum* cells[3]. This approach was observed for the immune response to human pythiosis when interleukin 4 and 5 production was detected in association with high IgE titers; a large amount of inflammatory cells (eosinophils and mast cells) was identified, which indicated a Th2 response during the infection. After immunotherapy, the patients presented high blood levels of interleukin 2 and INF-γ with a mononuclear immune response, which is typical of a Th1 response[18,19]. Additionally, an increase in the enzyme activity of ecto-adenosine deaminase (E-ADA) was observed in a rabbit model of experimental pythiosis, which is also associated with the switch from a Th2 to a Th1 response[123].

Despite the curative properties of this type of immunotherapy, the antibodies that are produced do not prevent host reinfection[2,115]. Santos *et al*[115] described a case of reinfection that occurred two years after the end of a successful immunotherapy treatment against pythiosis. Reinfection occurred at a different anatomical site than the initial infection (abdomen versus left pelvic limb), and although the new lesion was larger (60 cm perilesional edema and ulcerated lesions with approximately 20 cm in diameter), a cure was achieved with four immunotherapy doses (versus the five doses needed in the primary treatment). It is important to note that the levels of antibody's anti-*P. insidiosum* are associated with the response to treatment. Antibody titers are stable or increase in cases of unsuccessful treatment or when there is a persistent or recurrent infection. In cases of healing, substantial reductions of antibody's titers are seen during the subsequent months after the resolution of the infection[35].

Given the above information, we can conclude that effective immunotherapy treatment can be obtained in association with a rapid and accurate diagnosis, and it may or may not be associated with surgical excision.

CONCLUSION

In summary, although the current immunotherapies used for treating pythiosis make use of crude *P. insidiosum* antigens, some studies have described the identification of immunodominant antigens[124,125], and the best aspects of these immunotherapeutic elements could lead to a new vaccination strategy that is more effective and protective. A recent description of the *P. insidiosum* transcriptome[57] uncovered many putative virulence proteins, and it provided a set of candidate targets for the development of better pythiosis diagnosis and treatment modalities. Because the production of IgG by stimulated B cells is known to protect the host for short periods of time[2,115], the development of effective adjuvants and new diagnostic techniques for early disease diagnosis are of utmost importance, primarily for animal and human use in endemic areas.

REFERENCES

1 **Beakes GW**, Glockling SL, Sekimoto S. The evolutionary phylogeny of the oomycete "fungi". *Protoplasma* 2012; **249**: 3-19 [PMID: 21424613 DOI: 10.1007/s00709-011-0269-2]

2 **Gaastra W**, Lipman LJ, De Cock AW, Exel TK, Pegge RB, Scheurwater J, Vilela R, Mendoza L. Pythium insidiosum: an overview. *Vet Microbiol* 2010; **146**: 1-16 [PMID: 20800978 DOI: 10.1016/j.vetmic.2010.07.019]

3 **Mendoza L**, Newton JC. Immunology and immunotherapy of the infections caused by Pythium insidiosum. *Med Mycol* 2005; **43**: 477-486 [PMID: 16320491 DOI: 10.1080/13693780500279882]

4 **Fonseca AO**, Botton Sde A, Nogueira CE, Corrêa BF, Silveira Jde S, de Azevedo MI, Maroneze BP, Santurio JM, Pereira DI. In vitro reproduction of the life cycle of Pythium insidiosum from kunkers' equine and their role in the epidemiology of pythiosis. *Mycopathologia* 2014; **177**: 123-127 [PMID: 24326464 DOI: 10.1007/s11046-013-9720-6]

5 **Mendoza L**, Hernandez F, Ajello L. Life cycle of the human and animal oomycete pathogen Pythium insidiosum. *J Clin Microbiol* 1993; **31**: 2967-2973 [PMID: 8263182]

6 **Vanittanakom N**, Szekely J, Khanthawong S, Sawutdeechaikul P, Vanittanakom P, Fisher MC. Molecular detection of Pythium insidiosum from soil in Thai agricultural areas. *Int J Med Microbiol* 2014; **304**: 321-326 [PMID: 24444720 DOI: 10.1016/j.ijmm.2013.11.016]

7 **Santos CEP**, Santurio JM, Marques LC. Pythiosis of livestock in the Pantanal, Mato Grosso, Brazil. *Pesq Vet Bras* 2011; **31**: 1083-1089 [DOI: 10.1590/S0100-736X2011001200008]

8 **Krajaejun T**, Sathapatayavongs B, Pracharktam R, Nitiyanant P, Leelachaikul P, Wanachiwanawin W, Chaiprasert A, Assanasen P, Saipetch M, Mootsikapun P, Chetchotisakd P, Lekhakula A, Mitarnun W, Kalnauwakul S, Supparatpinyo K, Chaiwarith R, Chiewchanvit S, Tananuvat N, Srisiri S, Suankratay C, Kulwichit W, Wongsaisuwan M, Somkaew S. Clinical and epidemiological analyses of human pythiosis in Thailand. *Clin Infect Dis* 2006; **43**: 569-576 [PMID: 16886148 DOI: 10.1086/506353]

9 **Santos CEP**, Ubiali DG, Pescador CA, Zanette RA, Santurio JM, Marques LC. Epidemiological survey of equine pythiosis in the Brazilian Pantanal and nearby areas: results of 76 Cases. *J Equine Vet Sci* 2014; **34**: 270-274 [DOI: 10.1016/j.jevs.2013.06.003]

10 **Mendoza L**. Pythium insidiosum and mammalian hosts. In: Lamour K, Kamoun S, eds. Oomycete Genetics and Genomics: Diversity, Interactions and Research Tools. Hoboken, N.J.: John Wiley and Sons, 2009: 387-405

11 **Mendoza L**, Vilela R. Anomalous fungal and fungal-like infections: lacaziosis, pythiosis, and rhinosporidiosis. In: Anaissie EJ, McGinnis MR, Pfaller MA, editors. Clinical mycology. Edinburgh: Churchill Livingstone/Elsevier, 2009: 403-415

12 **Ravishankar JP**, Davis CM, Davis DJ, MacDonald E, Makselan SD, Millward L, Money NP. Mechanics of solid tissue invasion by the mammalian pathogen Pythium insidiosum. *Fungal Genet Biol* 2001; **34**: 167-175 [PMID: 11728155 DOI: 10.1006/fgbi.2001.1304]

13 **Schurko A**, Mendoza L, de Cock AW, Klassen GR. Evidence for geographic clusters: Molecular genetic differences among strains of Pythium insidiosum from Asia, Australia and the Americas are explored. *Mycologia* 2003; **95**: 200-208 [PMID:

21156606]
14 **Hussein MR**. Mucocutaneous Splendore-Hoeppli phenomenon. *J Cutan Pathol* 2008; **35**: 979-988 [PMID: 18976399 DOI: 10.1111/j.1600-0560.2008.01045.x]
15 **Miller RI**, Campbell RS. The comparative pathology of equine cutaneous phycomycosis. *Vet Pathol* 1984; **21**: 325-332 [PMID: 6730223]
16 **Martins TB**, Kommers GD, Trost ME, Inkelmann MA, Fighera RA, Schild AL. A comparative study of the histopathology and immunohistochemistry of pythiosis in horses, dogs and cattle. *J Comp Pathol* 2012; **146**: 122-131 [PMID: 21824626 DOI: 10.1016/j.jcpa.2011.06.006]
17 **Mendoza L**, Mandy W, Glass R. An improved Pythium insidiosum-vaccine formulation with enhanced immunotherapeutic properties in horses and dogs with pythiosis. *Vaccine* 2003; **21**: 2797-2804 [PMID: 12798620 DOI: 10.1016/S0264-410x(03)00225-1]
18 **Wanachiwanawin W**, Mendoza L, Visuthisakchai S, Mutsikapan P, Sathapatayavongs B, Chaiprasert A, Suwanagool P, Manuskiatti W, Ruangsetakit C, Ajello L. Efficacy of immunotherapy using antigens of Pythium insidiosum in the treatment of vascular pythiosis in humans. *Vaccine* 2004; **22**: 3613-3621 [PMID: 15315840 DOI: 10.1016/j.vaccine.2004.03.031]
19 **Thitithanyanont A**, Mendoza L, Chuansumrit A, Pracharktam R, Laothamatas J, Sathapatayavongs B, Lolekha S, Ajello L. Use of an immunotherapeutic vaccine to treat a life-threatening human arteritic infection caused by Pythium insidiosum. *Clin Infect Dis* 1998; **27**: 1394-1400 [PMID: 9868649]
20 **Álvarez JAC**, Viloria MIV, Ayola SCP. Clinical and histopathological evaluation of cutaneous pythiosis in donkeys (Equus asinus). *Rev Med Vet* (Bogota) 2013; **25**: 9-19
21 **Videla R**, van Amstel S, O'neill SH, Frank LA, Newman SJ, Vilela R, Mendoza L. Vulvar pythiosis in two captive camels (Camelus dromedarius). *Med Mycol* 2012; **50**: 219-224 [PMID: 21696258 DOI: 10.3109/13693786.2011.588970]
22 **Leal ABM**, Leal AT, Santurio JM, Kommers GD, Catto JB. Equine pythiosis in the Brazilian Pantanal region: Clinical and pathological findings of typical and atypical cases. *Pesq Vet Bras* 2001; **21**: 151-156
23 **Reis JL**, de Carvalho EC, Nogueira RH, Lemos LS, Mendoza L. Disseminated pythiosis in three horses. *Vet Microbiol* 2003; **96**: 289-295 [PMID: 14559176 DOI: 10.1016/j.vetmic.2003.07.005]
24 **Santos CEP**, Santurio JM, Colodel EM, Juliano RS, Silva JA, Marques LC. Contribution to the study of cutaneous pythiosis in equidae from northern Pantanal, Brazil. *Ars Vet* 2011; **27**: 134-140
25 **Humber RA**, Brown CC, Kornegay RW. Equine zygomycosis caused by Conidiobolus lamprauges. *J Clin Microbiol* 1989; **27**: 573-576 [PMID: 2715329]
26 **Santurio JM**, Monteiro AB, Leal AT, Kommers GD, de Sousa RS, Catto JB. Cutaneous Pythiosis insidiosi in calves from the Pantanal region of Brazil. *Mycopathologia* 1998; **141**: 123-125 [PMID: 9755503]
27 **Thomas RC**, Lewis DT. Pythiosis in dogs and cats. *Comp Cont Educ Pract Vet* 1998; **20**: 63-75
28 **Gabriel AL**, Kommers GD, Trost ME, Barros CSL, Pereira DB, Schwendler SE, Santurio JM. Outbreak of cutaneous pythiosis in cattle. *Pesq Vet Bras* 2008; **28**: 583-587
29 **Grecco FB**, Schild AL, Quevedo P, Assis-Brasil ND, Kommers GD, Marcolongo-Pereira C, Soares MP. Cutaneous pythiosis in cattle in the Southern region of Rio Grande do Sul, Brazil. *Pesq Vet Bras* 2009; **29**: 938-942
30 **Cardona Álvarez JA**, Vargas Viloria M, Perdomo SC. Frequency of presentation of bovine cutaneous pythiosis (Pythium insidiosum) in three cattle farms in Córdoba, Colombia. *CES Med Vet Zoot* 2012; **7**: 47-54
31 **Dykstra MJ**, Sharp NJ, Olivry T, Hillier A, Murphy KM, Kaufman L, Kunkle GA, Pucheu-Haston C. A description of cutaneous-subcutaneous pythiosis in fifteen dogs. *Med Mycol* 1999; **37**: 427-433 [PMID: 10647124]
32 **Schmiedt CW**, Stratton-Phelps M, Torres BT, Bell D, Uhl EW, Zimmerman S, Epstein J, Cornell KK. Treatment of intestinal pythiosis in a dog with a combination of marginal excision, chemotherapy, and immunotherapy. *J Am Vet Med Assoc* 2012; **241**: 358-363 [PMID: 22812473 DOI: 10.2460/javma.241.3.358]
33 **Fernandes CP**, Giordani C, Grecco FB, V Sallis ES, R Stainki D, Gaspar LF, Garcez Ribeiro CL, Nobre MO. Gastric pythiosis in a dog. *Rev Iberoam Micol* 2012; **29**: 235-237 [PMID: 22306044 DOI: 10.1016/j.riam.2012.01.002]
34 **Berryessa NA**, Marks SL, Pesavento PA, Krasnansky T, Yoshimoto SK, Johnson EG, Grooters AM. Gastrointestinal pythiosis in 10 dogs from California. *J Vet Intern Med* 2008; **22**: 1065-1069 [PMID: 18647164 DOI: 10.1111/j.1939-1676.2008.0123.x]
35 **Hensel P**, Greene CE, Medleau L, Latimer KS, Mendoza L. Immunotherapy for treatment of multicentric cutaneous pythiosis in a dog. *J Am Vet Med Assoc* 2003; **223**: 215-28, 197 [PMID: 12875449]
36 **Grooters AM**, Leise BS, Lopez MK, Gee MK, O'Reilly KL. Development and evaluation of an enzyme-linked immunosorbent assay for the serodiagnosis of pythiosis in dogs. *J Vet Intern Med* 2002; **16**: 142-146 [PMID: 11899028]
37 **Cooper RC**, Allison N, Boring JG. Apparent successful surgical treatment of intestinal pythiosis with vascular invasion in a dog. *Canine Pract* 1991; **16**: 9-12
38 **Foil CSO**, Short BG, Fadok VA, Kunkle GA. A report of subcutaneous pythiosis in 5 dogs and a review of the etiologic agent Pythium spp. *J Am Anim Hosp Assoc* 1984; **20**: 959-966
39 **Ader PL**. Phycomycosis in fifteen dogs and two cats. *J Am Vet Med Assoc* 1979; **174**: 1216-1223 [PMID: 438051]
40 **Tabosa IM**, Riet-Correa F, Nobre VM, Azevedo EO, Reis-Júnior JL, Medeiros RM. Outbreaks of pythiosis in two flocks of sheep in northeastern Brazil. *Vet Pathol* 2004; **41**: 412-415 [PMID: 15232143]
41 **Carrera MV**, Peixoto RM, Gouveia GV, Pessoa CRM, Jesus FPK, Santurio JM, Botton SA, Costa MM. Pythiosis in sheep from Pernambuco and Bahia States, Brazil. *Pesq Vet Bras* 2013; **33**: 476-482 [DOI: 10.1590/S0100-736X2013000400011]
42 **Camus AC**, Grooters AM, Aquilar RE. Granulomatous pneumonia caused by Pythium insidiosum in a central American jaguar, Panthera onca. *J Vet Diagn Invest* 2004; **16**: 567-571 [PMID: 15586573]
43 **Grooters AM**. Pythiosis, lagenidiosis, and zygomycosis in small animals. *Vet Clin North Am Small Anim Pract* 2003; **33**: 695-720, v [PMID: 12910739 DOI: 10.1016/S0195-5616(03)00034-2]
44 **Pesavento PA**, Barr B, Riggs SM, Eigenheer AL, Pamma R, Walker RL. Cutaneous pythiosis in a nestling white-faced ibis. *Vet Pathol* 2008; **45**: 538-541 [PMID: 18587102 DOI: 10.1354/vp.45-4-538]
45 **Badenoch PR**, Coster DJ, Wetherall BL, Brettig HT, Rozenbilds MA, Drenth A, Wagels G. Pythium insidiosum keratitis confirmed by DNA sequence analysis. *Br J Ophthalmol* 2001; **85**: 502-503 [DOI: 10.1136/bjo.85.4.496g]
46 **Murdoch D**, Parr D. Pythium insidiosum keratitis. *Aust N Z J Ophthalmol* 1997; **25**: 177-179 [PMID: 9267609 DOI: 10.1111/j.1442-9071.1997.tb01304.x]
47 **Virgile R**, Perry HD, Pardanani B, Szabo K, Rahn EK, Stone J, Salkin I, Dixon DM. Human infectious corneal ulcer caused by Pythium insidiosum. *Cornea* 1993; **12**: 81-83 [PMID: 8458239 DOI: 10.1097/00003226-199301000-00015]
48 **Bosco Sde M**, Bagagli E, Araújo JP, Candeias JM, de Franco MF, Alencar Marques ME, Mendoza L, de Camargo RP, Alencar Marques S. Human pythiosis, Brazil. *Emerg Infect Dis* 2005; **11**: 715-718 [PMID: 15890126 DOI: 10.3201/eid1105.040943]
49 **Shenep JL**, English BK, Kaufman L, Pearson TA, Thompson JW, Kaufman RA, Frisch G, Rinaldi MG. Successful medical

therapy for deeply invasive facial infection due to Pythium insidiosum in a child. *Clin Infect Dis* 1998; **27**: 1388-1393 [PMID: 9868648 DOI: 10.1086/515042]
50 **Triscott JA**, Weedon D, Cabana E. Human subcutaneous pythiosis. *J Cutan Pathol* 1993; **20**: 267-271 [PMID: 8366216 DOI: 10.1111/j.1600-0560.1993.tb00654.x]
51 **Thianprasit M**, Chaiprasert A, Imwidthaya P. Human pythiosis. *Curr Top Med Mycol* 1996; **7**: 43-54 [PMID: 9504058]
52 **Walker EM**, Walker SM. Effects of iron overload on the immune system. *Ann Clin Lab Sci* 2000; **30**: 354-365 [PMID: 11045759]
53 **Jurado RL**. Iron, infections, and anemia of inflammation. *Clin Infect Dis* 1997; **25**: 888-895 [PMID: 9356804 DOI: 10.1086/515549]
54 **Ekiz C**, Agaoglu L, Karakas Z, Gurel N, Yalcin I. The effect of iron deficiency anemia on the function of the immune system. *Hematol J* 2005; **5**: 579-583 [PMID: 15692603 DOI: 10.1038/sj.thj.6200574]
55 **Johnson L**. Iron and siderophores in fungal-host interactions. *Mycol Res* 2008; **112**: 170-183 [PMID: 18280720 DOI: 10.1016/j.mycres.2007.11.012]
56 **Krajaejun T**, Khositnithikul R, Lerksuthirat T, Lowhnoo T, Rujirawat T, Petchthong T, Yingyong W, Suriyaphol P, Smittipat N, Juthayothin T, Phuntumart V, Sullivan TD. Expressed sequence tags reveal genetic diversity and putative virulence factors of the pathogenic oomycete Pythium insidiosum. *Fungal Biol* 2011; **115**: 683-696 [PMID: 21724174 DOI: 10.1016/j.funbio.2011.05.001]
57 **Krajaejun T**, Lerksuthirat T, Garg G, Lowhnoo T, Yingyong W, Khositnithikul R, Tangphatsornruang S, Suriyaphol P, Ranganathan S, Sullivan TD. Transcriptome analysis reveals pathogenicity and evolutionary history of the pathogenic oomycete Pythium insidiosum. *Fungal Biol* 2014 [DOI: 10.1016/j.funbio.2014.01.009]
58 **Sathapatayavongs B**, Leelachaikul P, Prachaktam R, Atichartakarn V, Sriphojanart S, Trairatvorakul P, Jirasiritham S, Nontasut S, Eurvilaichit C, Flegel T. Human pythiosis associated with thalassemia hemoglobinopathy syndrome. *J Infect Dis* 1989; **159**: 274-280 [PMID: 2644370 DOI: 10.1093/infdis/159.2.274]
59 **González Charry H**, Trheebilcock Perna E, Montaño Aguirre J, León J. Potassium iodine (K.I.) as treatment for subcutaneous equine phycomycosis. *Revista ICA* (Colombia) 1979; **14**: 115-122
60 **Miller RI**, Wold D, Lindsay WA, Beadle RE, McClure JJ, McClure JR, McCoy DJ. Complications associated with immunotherapy of equine phycomycosis. *J Am Vet Med Assoc* 1983; **182**: 1227-1229 [PMID: 6863139]
61 **Morton LD**, Morton DG, Baker GJ, Gelberg HB. Chronic eosinophilic enteritis attributed to Pythium sp. in a horse. *Vet Pathol* 1991; **28**: 542-544 [PMID: 1771746 DOI: 10.1177/030098589102800615]
62 **Chaffin MK**, Schumacher J, Hooper N. Multicentric cutaneous pythiosis in a foal. *J Am Vet Med Assoc* 1992; **201**: 310-312 [PMID: 1500331]
63 **Worster AA**, Lillich JD, Cox JH, Rush BR. Pythiosis with bone lesions in a pregnant mare. *J Am Vet Med Assoc* 2000; **216**: 1795-1798, 1760 [PMID: 10844973]
64 **Santos CEP**, Juliano RS, Santurio JM, Marques LC. Efficacy of immunotherapy in the treatment of facial horse pythiosis. *Acta Sci Vet* 2011; **39**: 955
65 **Mosbah E**, Karrouf GIA, Younis EA, Saad HS, Ahdy A, Zaghloul AE. Diagnosis and surgical management of pythiosis in draft horses: report of 33 cases in Egypt. *J Equine Vet Sci* 2012; **32**: 164-169 [DOI: 10.1016/j.jevs.2011.08.014]
66 **Liljebjelke KA**, Abramson C, Brockus C, Greene CE. Duodenal obstruction caused by infection with Pythium insidiosum in a 12-week-old puppy. *J Am Vet Med Assoc* 2002; **220**: 1188-1191, 1162 [PMID: 11990966]
67 **Rakich PM**, Grooters AM, Tang KN. Gastrointestinal pythiosis in two cats. *J Vet Diagn Invest* 2005; **17**: 262-269 [PMID: 15945385 DOI: 10.1177/104063870501700310]
68 **Wellehan JF**, Farina LL, Keoughan CG, Lafortune M, Grooters AM, Mendoza L, Brown M, Terrell SP, Jacobson ER, Heard DJ. Pythiosis in a dromedary camel (Camelus dromedarius). *J Zoo Wildl Med* 2004; **35**: 564-568 [PMID: 15732604 DOI: 10.1638/03-098]
69 **Buergelt C**, Powe J, White T. Abdominal pythiosis in a Bengal tiger (Panthera tigris tigris). *J Zoo Wildl Med* 2006; **37**: 186-189 [PMID: 17312799 DOI: 10.1638/05-003.1]
70 **Santos SA**. Recomendações sobre manejo nutricional para equinos criados em pastagens nativas no Pantanal. Corumbá: EMBRAPA-CPAP, 1997
71 **Loreto ES**, Alves SH, Santurio JM, Nogueira CW, Zeni G. Diphenyl diselenide in vitro and in vivo activity against the oomycete Pythium insidiosum. *Vet Microbiol* 2012; **156**: 222-226 [PMID: 22055205 DOI: 10.1016/j.vetmic.2011.10.008]
72 **Zanette RA**, Bitencourt PE, Alves SH, Fighera RA, Flores MM, Wolkmer P, Hecktheuer PA, Thomas LR, Pereira PL, Loreto ES, Santurio JM. Insights into the pathophysiology of iron metabolism in Pythium insidiosum infections. *Vet Microbiol* 2013; **162**: 826-830 [PMID: 23182911 DOI: 10.1016/j.vetmic.2012.10.036]
73 **Santurio JM**, Ferreiro L. Pitiose: uma abordagem micológica e terapêutica. Porto Alegre: Editora da UFRGS, 2008
74 **Leal AT**, Leal ABM, Flores EF, Santurio JM. *Pythiosis. Cienc Rural* 2001; **31**: 735-743
75 **Grooters AM**, Whittington A, Lopez MK, Boroughs MN, Roy AF. Evaluation of microbial culture techniques for the isolation of Pythium insidiosum from equine tissues. *J Vet Diagn Invest* 2002; **14**: 288-294 [PMID: 12152807]
76 **Pereira DIB**, Santurio JM, Alves SH, Argenta JS, Cavalheiro AS, Ferreiro L. In vitro zoosporogenesis among oomycetes Pythium insidiosum isolates. *Cienc Rural* 2008; **38**: 143-147
77 **Grooters AM**, Gee MK. Development of a nested polymerase chain reaction assay for the detection and identification of Pythium insidiosum. *J Vet Intern Med* 2002; **16**: 147-152
78 **Schurko AM**, Mendoza L, de Cock AW, Bedard JE, Klassen GR. Development of a species-specific probe for Pythium insidiosum and the diagnosis of pythiosis. *J Clin Microbiol* 2004; **42**: 2411-2418 [PMID: 15184412]
79 **Vanittanakom N**, Supabandhu J, Khamwan C, Praparattanapan J, Thirach S, Prasertwitayakij N, Louthrenoo W, Chiewchanvit S, Tananuvat N. Identification of emerging human-pathogenic Pythium insidiosum by serological and molecular assay-based methods. *J Clin Microbiol* 2004; **42**: 3970-3974 [PMID: 15364977 DOI: 10.1128/Jcm.42.9.3970-3974.2004]
80 **Botton SA**, Pereira DI, Costa MM, Azevedo MI, Argenta JS, Jesus FP, Alves SH, Santurio JM. Identification of Pythium insidiosum by nested PCR in cutaneous lesions of Brazilian horses and rabbits. *Curr Microbiol* 2011; **62**: 1225-1229 [PMID: 21188592 DOI: 10.1007/s00284-010-9781-4]
81 **Thongsri Y**, Wonglakorn L, Chaiprasert A, Svobodova L, Hamal P, Pakarasang M, Prariyachatigul C. Evaluation for the clinical diagnosis of Pythium insidiosum using a single-tube nested PCR. *Mycopathologia* 2013; **176**: 369-376 [PMID: 23948967 DOI: 10.1007/s11046-013-9695-3]
82 **Miller RI**, Campbell RS. Immunological studies on equine phycomycosis. *Aust Vet J* 1982; **58**: 227-231 [PMID: 6814414]
83 **Witkamp J**. Bijdrage tot de kennis van de Hyphomycosis destruens. *Nederlandsh-Indisch blande voor Diergeneeskunde en Dierenteelt* 1924; **36**: 229-345
84 **Mendoza L**, Nicholson V, Prescott JF. Immunoblot analysis of the humoral immune response to Pythium insidiosum in horses with pythiosis. *J Clin Microbiol* 1992; **30**: 2980-2983 [PMID: 1452669]
85 **Mendoza L**, Kaufman L, Standard PG. Immunodiffusion test for diagnosing and monitoring pythiosis in horses. *J Clin Microbiol* 1986; **23**: 813-816 [PMID: 3086368]
86 **Imwidthaya P**, Srimuang S. Immunodiffusion test for diag-

nosing human pythiosis. *Mycopathologia* 1989; **106**: 109-112 [PMID: 2507920]
87 **Miller RI**, Campbell RS. Haematology of horses with phycomycosis. *Aust Vet J* 1983; **60**: 28-29 [PMID: 6830547]
88 **Mendoza L**, Alfaro AA. Equine pythiosis in Costa Rica: report of 39 cases. *Mycopathologia* 1986; **94**: 123-129 [PMID: 3088454]
89 **Krajaejun T**, Kunakorn M, Niemhom S, Chongtrakool P, Pracharktam R. Development and evaluation of an in-house enzyme-linked immunosorbent assay for early diagnosis and monitoring of human pythiosis. *Clin Diagn Lab Immunol* 2002; **9**: 378-382 [PMID: 11874882 DOI: 10.1128/Cdli.9.2.378-382.2002]
90 **Santurio JM**, Leal AT, Leal ABM, Alves SH, Lubeck I, Griebeler J, Copetti MV. Indirect ELISA for the serodiagnostic of pythiosis. *Pesq Vet Bras* 2006; **26**: 47-50
91 **Jindayok T**, Piromsontikorn S, Srimuang S, Khupulsup K, Krajaejun T. Hemagglutination test for rapid serodiagnosis of human pythiosis. *Clin Vaccine Immunol* 2009; **16**: 1047-1051 [PMID: 19494087 DOI: 10.1128/Cvi.00113-09]
92 **Krajaejun T**, Imkhieo S, Intaramat A, Ratanabanangkoon K. Development of an immunochromatographic test for rapid serodiagnosis of human pythiosis. *Clin Vaccine Immunol* 2009; **16**: 506-509 [PMID: 19225072 DOI: 10.1128/Cvi.00276-08]
93 **Supabandhu J**, Vanittanakom P, Laohapensang K, Vanittanakom N. Application of immunoblot assay for rapid diagnosis of human pythiosis. *J Med Assoc Thai* 2009; **92**: 1063-1071 [PMID: 19694332]
94 **Trost ME**, Gabriel AL, Masuda EK, Fighera RA, Irigoyen LF, Kommers GD. Clinical, morphologic and immunohistochemical aspects of canine gastrintestinal pythiosis. *Pesq Vet Bras* 2009; **29**: 673-679
95 **Salipante SJ**, Hoogestraat DR, SenGupta DJ, Murphey D, Panayides K, Hamilton E, Castañeda-Sánchez I, Kennedy J, Monsaas PW, Mendoza L, Stephens K, Dunn JJ, Cookson BT. Molecular diagnosis of subcutaneous Pythium insidiosum infection by use of PCR screening and DNA sequencing. *J Clin Microbiol* 2012; **50**: 1480-1483 [PMID: 22205808 DOI: 10.1128/JCM.06126-11]
96 **Mendoza L**, Prasla SH, Ajello L. Orbital pythiosis: a non-fungal disease mimicking orbital mycotic infections, with a retrospective review of the literature. *Mycoses* 2004; **47**: 14-23 [PMID: 14998394]
97 **Argenta JS**, Santurio JM, Alves SH, Pereira DI, Cavalheiro AS, Spanamberg A, Ferreiro L. In vitro activities of voriconazole, itraconazole, and terbinafine alone or in combination against Pythium insidiosum isolates from Brazil. *Antimicrob Agents Chemother* 2008; **52**: 767-769 [PMID: 18056274 DOI: 10.1128/Aac.01075-07]
98 **Pereira DI**, Santurio JM, Alves SH, Argenta JS, Pötter L, Spanamberg A, Ferreiro L. Caspofungin in vitro and in vivo activity against Brazilian Pythium insidiosum strains isolated from animals. *J Antimicrob Chemother* 2007; **60**: 1168-1171 [PMID: 17785281 DOI: 10.1093/Jac/Dkm332]
99 **Cavalheiro AS**, Zanette RA, Spader TB, Lovato L, Azevedo MI, Botton S, Alves SH, Santurio JM. In vitro activity of terbinafine associated to amphotericin B, fluvastatin, rifampicin, metronidazole and ibuprofen against Pythium insidiosum. *Vet Microbiol* 2009; **137**: 408-411 [PMID: 19269752 DOI: 10.1016/j.vetmic.2009.01.036]
100 **Cavalheiro AS**, Maboni G, de Azevedo MI, Argenta JS, Pereira DI, Spader TB, Alves SH, Santurio JM. In Vitro activity of terbinafine combined with caspofungin and azoles against Pythium insidiosum. *Antimicrob Agents Chemother* 2009; **53**: 2136-2138 [PMID: 19289531 DOI: 10.1128/Aac.01506-08]
101 **Loreto ES**, Mario DA, Denardi LB, Alves SH, Santurio JM. In vitro susceptibility of Pythium insidiosum to macrolides and tetracycline antibiotics. *Antimicrob Agents Chemother* 2011; **55**: 3588-3590 [PMID: 21537028 DOI: 10.1128/Aac.01586-10]
102 **Mahl DL**, de Jesus FP, Loreto É, Zanette RA, Ferreiro L, Pilotto MB, Alves SH, Santurio JM. In vitro susceptibility of Pythium insidiosum isolates to aminoglycoside antibiotics and tigecycline. *Antimicrob Agents Chemother* 2012; **56**: 4021-4023 [PMID: 22508303 DOI: 10.1128/Aac.00073-12]
103 **Miller RI**. Treatment of equine phycomycosis by immunotherapy and surgery. *Aust Vet J* 1981; **57**: 377-382 [PMID: 7342944]
104 **Mendoza L**, Villalobos J, Calleja CE, Solis A. Evaluation of two vaccines for the treatment of pythiosis insidiosi in horses. *Mycopathologia* 1992; **119**: 89-95 [PMID: 1435952]
105 **Miller RI**, Campbell RS. Clinical observations on equine phycomycosis. *Aust Vet J* 1982; **58**: 221-226 [PMID: 6890342]
106 **Santurio JM**, Leal AT, Leal AB, Festugatto R, Lubeck I, Sallis ES, Copetti MV, Alves SH, Ferreiro L. Three types of immunotherapics against pythiosis insidiosi developed and evaluated. *Vaccine* 2003; **21**: 2535-2540 [PMID: 12744888 DOI: 10.1016/S0264-410x(03)00035-5]
107 **Monteiro AB**. Immunotherapy of equine phytiosis: testing the efficacy of a biological and evaluation of the leukocytic response to the treatment in horses naturally infected with Pythium insidiosum. Master in Veterinary Medicine. Santa Maria, RS, Brazil: Federal University of Santa Maria, 1999
108 **Pereira DI**, Botton SA, Azevedo MI, Motta MA, Lobo RR, Soares MP, Fonseca AO, Jesus FP, Alves SH, Santurio JM. Canine gastrointestinal pythiosis treatment by combined antifungal and immunotherapy and review of published studies. *Mycopathologia* 2013; **176**: 309-315 [PMID: 23918089 DOI: 10.1007/s11046-013-9683-7]
109 **Mendoza L**, Alfaro AA, Villalobos J. Bone lesions caused by Pythium insidiosum in a horse. *J Med Vet Mycol* 1988; **26**: 5-12 [PMID: 3379540]
110 **Eaton SA**. Osseous involvement by Pythium insidiosum. *Comp Cont Educ Pract Vet* 1993; **15**: 485-488
111 **Fisher EM**. Cutaneous phycomycosis in two horses. *Aust Vet J* 2000; **78**: 257 [PMID: 10840572 DOI: 10.1111/j.1751-0813.1999.tb12942.x]
112 **Maciel ICD**, Silveira JT, Maia CA, Sousa RM, Oliveira NJF, Duarte ER. Fatal pythiosis in horse initially treated to cutaneous habronemiasis. *Acta Sci Vet* 2008; **36**: 293-297
113 **White SD**, Ghoddusi M, Grooters AM, Jones K. Cutaneous pythiosis in a nontravelled California horse. *Vet Dermatol* 2008; **19**: 391-394 [PMID: 18699814 DOI: 10.1111/j.1365-3164.2008.00690.x]
114 **Bandeira A**, Santos JdA, Melo Cd, Andrade V, Dantas A, Araujo J. Equine cutaneous pythiosis in Sergipe State, Brazil. *Cienc Vet Tróp* 2009; **12**: 46-54
115 **Santos CE**, Marques LC, Zanette RA, Jesus FP, Santurio JM. Does immunotherapy protect equines from reinfection by the oomycete Pythium insidiosum? *Clin Vaccine Immunol* 2011; **18**: 1397-1399 [PMID: 21715582 DOI: 10.1128/Cvi.05150-11]
116 **Laohapensang K**, Rerkasem K, Supabandhu J, Vanittanakom N. Necrotizing arteritis due to emerging Pythium insidiosum infection in patients with thalassemia: rapid diagnosis with PCR and serological tests-case reports. *Int J Angiol* 2005; **14**: 123-128 [DOI: 10.1007/s00547-005-2012-3]
117 **Pupaibool J**, Chindamporn A, Patrakul K, Suankratay C, Sindhuphak W, Kulwichit W. Human pythiosis. *Emerg Infect Dis* 2006; **12**: 517-518 [PMID: 16710978]
118 **Lekhanont K**, Chuckpaiwong V, Chongtrakool P, Aroonroch R, Vongthongsri A. Pythium insidiosum keratitis in contact lens wear: a case report. *Cornea* 2009; **28**: 1173-1177 [PMID: 19730096 DOI: 10.1097/ICO.0b013e318199fa41]
119 **Sudjaritruk T**, Sirisanthana V. Successful treatment of a child with vascular pythiosis. *BMC Infect Dis* 2011; **11**: 33 [PMID: 21276255 DOI: 10.1186/1471-2334-11-33]
120 **Keoprasom N**, Chularojanamontri L, Chayakulkeeree M, Chaiprasert A, Wanachiwanawin W, Ruangsetakit C. Vascular pythiosis in a thalassemic patient presenting as bilateral leg ulcers. *Med Mycol Case Rep* 2012; **2**: 25-28 [PMID:

24432209 DOI: 10.1016/j.mmcr.2012.12.002]
121 **Schloemer NJ**, Lincoln AH, Mikhailov TA, Collins CL, Di Rocco JR, Kehl SC, Chusid MJ. Fatal disseminated Pythium insidiosum infection in a child with Diamond-Blackfan anemia. *Infect Dis Clin Pract* 2013; **21**: e24-e26 [DOI: 10.1097/IPC.0b013e318278f3b5]
122 **Thanathanee O**, Enkvetchakul O, Rangsin R, Waraasawapati S, Samerpitak K, Suwan-apichon O. Outbreak of Pythium keratitis during rainy season: a case series. *Cornea* 2013; **32**: 199-204 [PMID: 22902492 DOI: 10.1097/Ico.0b013e3182535841]
123 **Bach BC**, Leal DB, Jaques JA, Souza Vdo C, Ruchel JB, Schlemmer KB, Zanette RA, Heckstheuer PA, de Lima Pereira P, Casali EA, Alves SH, Santurio JM. E-ADA activity in lymphocytes of an experimental model of pythiosis treated with immunotherapy. *Cell Biochem Funct* 2013; **31**: 476-481 [PMID: 23086808 DOI: 10.1002/cbf.2921]
124 **Krajaejun T**, Kunakorn M, Pracharktam R, Chongtrakool P, Sathapatayavongs B, Chaiprasert A, Vanittanakom N, Chindamporn A, Mootsikapun P. Identification of a novel 74-kiloDalton immunodominant antigen of Pythium insidiosum recognized by sera from human patients with pythiosis. *J Clin Microbiol* 2006; **44**: 1674-1680 [PMID: 16672392 DOI: 10.1128/Jcm.44.5.1674-1680.2006]
125 **Krajaejun T**, Keeratijarut A, Sriwanichrak K, Lowhnoo T, Rujirawat T, Petchthong T, Yingyong W, Kalambaheti T, Smittipat N, Juthayothin T, Sullivan TD. The 74-kilodalton immunodominant antigen of the pathogenic oomycete Pythium insidiosum is a putative exo-1,3-beta-glucanase. *Clin Vaccine Immunol* 2010; **17**: 1203-1210 [PMID: 20237199 DOI: 10.1128/Cvi.00515-09]

P- Reviewer: dos Santos CEP, Prariyachatigul C, Sahu RP, Wang ZX **S- Editor**: Ji FF **L- Editor**: A **E- Editor**: Wang CH

World Journal of Immunology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5411/wji.v4.i2.98

World J Immunol 2014 July 27; 4(2): 98-106
ISSN 2219-2824 (online)

REVIEW

GM3-containing nanoparticles in immunosuppressed hosts: Effect on myeloid-derived suppressor cells

Audry Fernández, Liliana Oliver, Rydell Alvarez, Luis E Fernández, Circe Mesa

Audry Fernández, Liliana Oliver, Rydell Alvarez, Circe Mesa, Immunobiology Division, Center of Molecular Immunology, Havana 11600, Cuba
Luis E Fernández, Innovation Division, Center of Molecular Immunology, Havana 11600, Cuba
Author contributions: Fernández A, Oliver L, Alvarez R, Fernández LE and Mesa C drafted the review and wrote the paper.
Supported by Center of Molecular Immunology
Correspondence to: Circe Mesa, PhD, Director of Immunobiology Division, Center of Molecular Immunology, 216 St. and 15th Avenue, Atabey, Playa, PO Box 16040, Havana 11600, Cuba. circe@cim.sld.cu
Telephone: +53-7-2143161 Fax: +53-7-2720644
Received: March 8, 2014 Revised: June 12, 2014
Accepted: June 27, 2014
Published online: July 27, 2014

Abstract

Cancer vaccines to date have not broadly achieved a significant impact on the overall survival of patients. The negative effect on the immune system of the tumor itself and conventional anti-tumor treatments such as chemotherapy is, undoubtedly, a key reason for these disappointing results. Myeloid-derived suppressor cells (MDSCs) are considered a central node of the immunosuppressive network associated with tumors. These cells inhibit the effector function of natural killer and $CD8^+$ T cells, expand regulatory T cells and can differentiate into tumor-associated macrophages within the tumor microenvironment. Thus, overcoming the suppressive effects of MDSCs is likely to be critical for cancer immunotherapy to generate effective anti-tumor immune responses. However, the capacity of cancer vaccines and particularly their adjuvants to overcome this inhibitory population has not been well characterized. Very small size proteoliposomes (VSSP) is a nanoparticulated adjuvant specifically designed to be formulated with vaccines used in the treatment of immunocompromised patients. This adjuvant contains immunostimulatory bacterial signals together with GM3 ganglioside. VSSP promotes dendritic cell maturation, antigen cross-presentation to $CD8^+$ T cells, Th1 polarization, and enhances $CD8^+$ T cell response in tumor-free mice. Currently, four cancer vaccines using VSSP as the adjuvant are in Phase Ⅰ and Ⅱ clinical trials. In this review, we summarize our work characterizing the unique ability of VSSP to stimulate antigen-specific $CD8^+$ T cell responses in two immunocompromised scenarios; in tumor-bearing mice and during chemotherapy-induced leukopenia. Particular emphasis has been placed on the interaction of these nanoparticles with MDSCs, as well as comparison with other cancer vaccine adjuvants currently in preclinical or clinical studies.

Key words: Very small size proteoliposomes; Adjuvants; Tumors; Myeloid-derived suppressor cells; Leukopenia; Chemotherapy

Core tip: Very small size proteoliposomes (VSSP) is a nanoparticulated adjuvant being used in the formulation of several cancer vaccines that are currently in clinical trials. In this review we summarize the unique ability of VSSP to stimulate antigen-specific $CD8^+$ T cell responses in tumor-bearing mice and in mice with chemotherapy-induced leukopenia, both immunosuppressive scenarios frequently found in cancer patients. As a possible mechanism of this efficacy, we have focused on the modulation of myeloid-derived suppressor cells (MDSCs) by these nanoparticles, in the context of the current knowledge about the interaction of cancer vaccine adjuvants with MDSCs.

Fernández A, Oliver L, Alvarez R, Fernández LE, Mesa C. GM3-containing nanoparticles in immunosuppressed hosts: Effect on myeloid-derived suppressor cells. *World J Immunol* 2014; 4(2): 98-106 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i2/98.htm DOI: http://dx.doi.org/10.5411/wji.v4.i2.98

INTRODUCTION

The central importance and complexity of the interactions between tumors and the immune system has only recently been recognized, with rapidly expanding investigations in the last decade. Tumors are not only shaped by the immune system[1,2] but actively induce impairment of antigen-presenting cells (APCs) as well as effector T lymphocytes[3,4], contributing significantly to both tumor progression and metastasis. One of the key cellular mediators of tumor-induced immunosuppression are myeloid-derived suppressor cells (MDSCs), which not only are the manifestation of the myeloid differentiation block that causes loss of mature APCs, but also actively and directly inhibit the lytic activity of both $CD8^+$ T cells[5,6] and NK cells[7].

MDSCs are currently thought of as a heterogeneous population of immature myeloid cells with suppressive activity. In mice these cells are routinely identified by the co-expression of CD11b and Gr1 markers. More recently, two subpopulations of MDSCs have been identified with different phenotypes and mechanisms of suppression: monocytic (Mo-MDSCs) and granulocytic (G-MDSCs)[8-11]. In tumor-bearing mice, as well as in cancer patients, the G-MDSCs constitute 70%-80% of overall MDSCs, whereas Mo-MDSCs represent only 20%-30%[11-14]. Mo-MDSCs ($CD11b^+Ly6C^{hi}Ly6G^-$) are highly immunosuppressive and exert their suppression *via* antigen-independent mechanisms[15-18]. In comparison, G-MDSCs ($CD11b^+Ly6C^{lo}Ly6G^+$) are moderately immunosuppressive, release reactive oxygen species (ROS) and require antigen-specific interaction with T cells to induce tolerance[9,11,19,20]. Several mechanisms of MDSC-mediated suppression have been described and are extensively detailed in other reviews[3,21]. Among these, the depletion of L-arginine, production of nitric oxide (NO) and generation of ROS/reactive nitrogen species have been linked to the overexpression of arginase 1 (ARG1), inducible nitric oxide synthase (NOS2) and NADPH oxidase[3,13,22]. MDSCs are also able to expand regulatory T cells (Tregs) populations[23,24] and can differentiate into tumor-associated macrophages within the tumor microenvironment[25,26]-both regulatory populations that play an important role in tumor-induced immunosuppression. Recent findings suggest that MDSCs can also facilitate tumor-progression and metastasis by increasing angiogenesis[27,28], *via* secretion of matrix metallopeptidases[29,30] and by aiding in the formation of the metastatic niche[27,31].

Given the pro-tumor importance of MDSCs, many efforts have been undertaken to find drugs capable of reducing the number of circulating MDSCs, abrogate MDSCs suppressive function or differentiate these cells into mature APCs. For instance, it has been demonstrated that 25-hydroxy vitamin D3 and all-trans retinoic acid reduce the frequency of MDSCs by inducing their differentiation towards $HLA\text{-}DR^+$ cells and dendritic cells (DCs), respectively, in patients with advance head and neck squamous cell carcinoma and metastatic renal cell carcinoma (RCC)[32-34]. Sunitinib, a pan-receptor tyrosine kinase inhibitor, and chemotherapeutic agents (taxanes, gemcitabine and 5-fluorouracil) also decrease circulating MDSCs in patients with RCC, melanoma, pancreatic and esophagogastric cancer[35,36]. Finally, the phosphodiesterase-5 inhibitor sildenafil diminishes the suppressive function of human MDSCs[37].

Although the pharmacological modulation of MDSCs represents a potentially important strategy for cancer treatment, none of these drugs detailed above have thus far improved the clinical outcome in cancer patients. These data suggest that inhibiting MDSCs alone (unlike the T cell checkpoint inhibitors) is not sufficient to achieve an effective anti-tumor response, and that combination with strategies to specifically activate immune responses against the cancer are needed. However, most cancer vaccines have not shown significant objective responses in clinical trials. But, the unimpressive clinical impact of active immunotherapy in cancer patients may be in turn tied to the immunosuppressive environment generated by tumors[3,4,21] as well as the aggressive chemotherapeutic treatments used in patients, which frequently induce leukopenia[38-40]. Thus, the combination of cancer vaccines with agents interfering with MDSCs number/function may be an effective approach to generate fully functional tumor-specific immune effectors. Even more desirable would be to find agents that are capable of simultaneously activating tumor-specific effector cells, inhibiting the suppressive function of MDSCs, and diminishing leukopenic period after chemotherapy. As detailed below, these are all properties of the VSSP adjuvant.

Adjuvants are critical but largely unappreciated components of vaccine formulations, necessary to potentiate the immune response specific for the nominal antigen. This is particularly important in cancer, where the vaccine antigen is often a self protein for which self-tolerance needs to be broken. In recent years the interaction of adjuvants with regulatory cells, and particularly MDSCs, have begun to be study[41-45]. This field is still in its infancy however, and there is only strong evidence for the modulation of tumor-induced MDSCs by synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG)[44], formalin-inactivated Herpes Simplex Virus[43] and VSSP[42], while indirect evidence suggests that other adjuvants may expand MDSCs once inoculated in the hosts. Therefore, the selection of suitable adjuvants for cancer vaccines is a very complex matter, and needs to be based in the ability to overcome the immunosuppression generated by tumors and chemotherapy. In this review we summarize the immunomodulatory properties of VSSP, a novel adjuvant for cancer immunotherapy.

GENERAL PROPERTIES OF VSSP

VSSP is a nanoparticulated adjuvant obtained through the hydrophobic incorporation of the GM3 ganglioside into outer membrane vesicles (OMPs) from *Neisseria meningitidis*[46]. It has been shown that VSSP contains TLR4

Table 1 Modulation caused by very small size proteoliposomes on different immune cell populations

Immune cell	Effect of VSSP	Ref.
DCs	Increases costimulation and MHCII expression	[47]
	Enhances production of IL-12, IL-6, IL-18, IL-1β and reduces secretion of IL-10	[47,48]
	Induces Th1-polarizing capacity	[47]
	Facilitates cross-presentation of protein antigens	[50]
MDSCs	Expands poorly suppressive MDSCs	[42]
	Reduces the suppressive function of tumor-induced MDSCs	[42]
	Impairs migration of tumor-induced MDSCs towards the tumor microenvironment	[42]
	Promotes differentiation of tumor-induced MDSCs into mature DCs	[42,59]
	Reduces the suppressive function of MDSCs generated during chemotherapy-induced leukopenia	[62]
$CD4^+$ T cells	Induces Th1 polarization	[47]
CTL	Potentiates CTL responses in healthy mice. Primary expansion independent of $CD4^+$ T cell help	[50]
	Generates similar CTL responses in tumor-free and tumor-bearing mice	[42]
	Increases $CD8^+$ T cell counts, with memory phenotype, and protects CTL response in leukopenic mice	[62]

VSSP: Very small size proteoliposomes; MDSCs: Myeloid-derived suppressor cells; DCs: Dendritic cells; CTL: Cytotoxic T lymphocytes.

and TLR2 ligands, which play an important role in the immunomodulatory properties of this compound[47,48]. Immunization of mice, monkeys and humans with VSSP generated IgM and IgG antibodies specific for both GM3 and OMPs[46,49]. This adjuvant also induced DC maturation, as evidenced by the increased expression of MHCII and CD40, CD80 and CD86 costimulatory molecules (Table 1)[47]. Additionally, VSSP-treated DCs secreted inflammatory cytokines such as IL-12p40/70 and IL-6[47]. DCs from healthy donors treated *in vitro* with VSSP produced not only higher levels of IL-6 but also decreased amount of IL-10, in comparison to lipopolysaccharide [LPS, the prototypic TLR4 agonist (Table 1)][48]. Experiments with antigen-specific transgenic T cells demonstrated that VSSP-treated DCs induced a Th1 phenotype in stimulated naïve $CD4^+$ T cells[47]. Furthermore, VSSP expanded $CD8^+$ T cells specific for the co-injected antigen and promoted an effective *in vivo* cytotoxic T lymphocytes (CTL) response[50]. In the latter case, $CD8^+$ T cell activation was mediated by the cross-presentation of exogenous antigens and did not require help from $CD4^+$ T cells (Table 1)[50].

More recently, we have found that VSSP treatment of naive mice (without a vaccine antigen) significantly increased the frequency of splenic $CD11b^+Gr1^+$ cells[42]. However, these $CD11b^+Gr1^+$ cells were poorly suppressive on both antigen-specific and allogeneic CTL assays (Table 1). The residual suppressive capacity of VSSP-derived MDSCs depended on NOS but not ARG, which was associated with a significant increase of NOS3 enzyme. Although VSSP contains TLR2 and TLR4 ligands, the interaction of these particles with the immune system appears to be more complex than can be explained by just TLR activation. For example, OMPs containing the same TLR ligands induced a significantly lower expansion of $CD11b^+Gr1^+$ cells than did VSSP, indicating that the presence of the GM3 ganglioside is also relevant for the immunomodulatory properties of this compound.

VSSP-induced expansion of MDSC numbers is not entirely unexpected, as MDSCs have also been reported to accumulate in mice treated with granulocyte and macrophage colony-stimulating factor (GM-CSF)[51,52], LPS[41], CpG[53], complete Freund's adjuvant[45] and Bacillus Calmette-Guérin from *Mycobacterium bovis*[54]. Similar MDSCs expansion has been described for other conditions involving major inflammatory responses, such as superantigen vaccination[55], polymicrobial sepsis[56], after burn[57] and traumatic injuries[58]. These findings are consistent with a physiological role of MDSCs as a counterbalancing mechanism to inflammation, preventing collateral damage to the tissue caused by activated T cells once the "dangerous" antigen has been eliminated.

EFFECT OF VSSP ON TUMOR-BEARING IMMUNOCOMPROMISED HOSTS

The effect of VSSP on the phenotype, suppressive function and differentiation status of tumor-induced MDSCs has been evaluated in mice bearing C26GM, EL4, EG.7 and MCA203 tumors (Table 1)[42]. Splenic MDSCs derived from VSSP-treated tumor-bearing mice (MDSCs-T+V) contained a higher frequency of $CD11b^+Gr1^{hi}$ and $Ly6C^{lo}Ly6G^+$ G-MDSCs than untreated tumor-bearing counterparts (MDSCs-T). In addition, IL-4Rα is downregulated on MDSCs-T+V, and these cells showed an increase of the homing molecule CD62L. Consistent with our *in vitro* studies, the suppressive function of tumor-induced splenic MDSCs was significantly reduced when VSSP is given *in vivo*. Several different findings support this effect of VSSP. First, MDSCs-T+V were unable to suppress the hemagglutinin (HA) peptide-specific proliferation of $CD8^+$ T cells from CL4 TCR transgenic mice, in the same experimental setting where equal number of MDSCs-T were significantly inhibitory. *In vitro* ^{51}Cr release CTL assays demonstrated that, as expected, MDSCs-T completely suppressed both antigen-specific and alloantigen-specific lytic activity of $CD8^+$ T cells. In contrast, MDSCs-T+V isolated from EL4 and C26GM tumor-bearing mice only marginally affected the generation of the CTL.

The effect of VSSP on MDSCs *in vivo* was further examined in adoptive transfer experiments. In the first approach, MDSCs-T and MDSC-T+V were adoptively transferred into $CD45.1^+$ B6 congenic mice, which previously received the transference of ovalbumin (OVA)-specific $CD8^+$ T cells from OTI transgenic mice, and vaccinated with the immunodominant $OVA_{257-264}$ (SIINFEKL) peptide emulsified in incomplete Freund's adju-

vant (IFA). Similar frequencies of IFN-γ$^+$ antigen-specific CD8$^+$ T cells were found in recipient mice transferred with MDSCs-T+V compared to control mice receiving no MDSCs, whereas transfer of MDSCs-T significantly impaired the activation of OTI lymphocytes. Additional experiments were performed to compare VSSP with other adjuvants or well-established vaccination systems. On this regard, we found that VSSP-based vaccines are more efficient than vaccination with DCs or vaccines employing the adjuvant polyinosinic:polycytidylic acid (polyI:C) in activating antigen-specific CTL responses in the presence of MDSCs-T. In fact, vaccination of BALB/c mice, which had been adoptively transferred with both congenic antigen-specific CD8$^+$ T cells and MDSCs-T, with HA peptide in VSSP adjuvant prevented the MDSCs-T-mediated suppression of CD8$^+$ T cell responses that was observed in mice vaccinated with HA-pulsed DCs. Also congenic OTI CD8$^+$ T cells transferred to EG.7 tumor-bearing mice produce IFN-γ in response to VSSP admixed with SIINFEKL peptide- but not to a vaccine consisting of SIINKEKL-pulsed DCs. Importantly, the OVA-specific *in vivo* CTL response generated in mice with EL4 tumors by the administration of OVA/VSSP was comparable to that observed in tumor-free mice, whereas vaccination with OVA/polyI:C was unable to overcome the tumor-induced impairment of the CTL response.

In addition to TCR transgenic T cell responses to a model antigen, we have found that VSSP blunts MDSC-mediated suppression of endogenous T cell responses to native tumor antigen, by measuring the inhibition of tumor-specific CD8$^+$ T cells by MDSCs in an ELISPOT assay. CD8$^+$ T cells isolated from MCA203 tumor-bearing mice did not release IFN-γ when stimulated with MCA203 tumor cells, irrespective of the presence of MDSCs. In contrast, a significant frequency of CD8$^+$ T cells derived from VSSP-treated tumor-bearing mice were activated by tumor cells and produced IFN-γ, even when MDSCs-T+V were added to the culture. Importantly, MDSCs-T maintained their ability to suppress tumor-specific CTL in this experiment.

Within the tumor microenvironment itself, VSSP treatment did not change the phenotype and functional capacity of CD11b$^+$ sorted MDSCs. However, adoptively transferred congenic MDSCs-T had a reduced ability to infiltrate tumors in EL4 tumor-bearing mice treated with VSSP. More importantly, in these VSSP-treated mice, tumor-infiltrating transferred MDSCs-T were more differentiated into CD11c$^+$MHCII$^+$CD11b$^-$ phenotype characteristic of DCs, and did not differentiate towards MHCII$^+$F4/80$^+$ macrophages. A similar differentiation pattern was observed *in vivo* in the spleen and lymph nodes from VSSP-inoculated tumor-bearing mice. In a more recent work, it was demonstrated that *in vitro* treatment with VSSP of tumor-induced MDSCs was sufficient to differentiate this immature population towards phenotypically mature DCs and, more importantly, causes the loss of their suppressive function[59]. Since VSSP contains a TLR4 ligand, a comparison with LPS was done in the same experimental setting. Interestingly, incubation with LPS fails to differentiate tumor-induced MDSCs into DCs and, consequently, these cells retain their inhibitory activity[59]. In agreement with these results, Greifenberg *et al*[60] have shown that incubation of bone marrow (BM)-derived MDSCs with the combination of LPS and IFN-γ increases NO secretion, enhancing the suppressive activity of these MDSCs and impairing their maturation into DCs. These findings further suggest that VSSP's effect on MDSCs is not a shared characteristic of all TLR4 agonists, but is a unique property of VSSP. Other authors have reported that TLR4 signaling is involved in the promotion of tumor growth associated with the recruitment of G-MDSCs, through the interaction with S100A9 protein[61]. VSSP also expands G-MDSCs subpopulation in tumor-bearing mice, however it also potentiates CTL responses and anti-tumor activity on those mice[42]. Therefore, the complexity of signals in the structure of VSSP (TLR2 agonist, GM3 ganglioside, *etc.*) likely makes these particles distinct from single TLR4 agonists. In fact, VSSP can induce activation of BM-derived DCs obtained from LPS hyporesponsive mice (C3H/HeJ)[47].

It has been shown in the literature that other adjuvants can also reduce the suppressive function of tumor-recruited MDSCs. For instance, intratumoral injection of CpG reduces the suppressive function of Mo-MDSCs and induces their differentiation towards macrophages with tumoricidal capability[44]. However, CpG does not modify G-MDSCs, and intratumoral injections in patients may be difficult to impossible. Formalin-inactivated Herpes Simplex Virus also decreases the suppressive function of MDSCs-T, but whether this adjuvant is able to differentiate MDSCs has not been addressed[43].

INFLUENCE OF VSSP ON CHEMOTHERAPY-ASSOCIATED IMMUNOSUPPRESSED HOSTS

The ability of VSSP to rescue the number and functionality of relevant immune populations on mice undergoing chemotherapy-induced leukopenia has been also tested (Table 1)[62]. The widely used chemotherapy agent cyclophosphamide (CY) was used to induce the leukopenic setting for these studies. In this model, VSSP accelerated the recovery of specific leukocytes population when administered in the early stages of leukopenia. Splenic CD4$^+$ and CD8$^+$ T cells (with a memory CD4$^+$CD44^{hi} and CD8$^+$CD44^{hi} phenotype) and CD11c$^+$CD11b$^+$ DCs were some of the populations most enhanced by VSSP in leukopenic mice. Interestingly, MDSCs were also significantly expanded. However, similar to what was seen in the tumor-mediated immunosuppression setting, MDSCs from leukopenic mice treated with VSSP showed a reduced capacity to suppress T cell responses, compared to CY-induced MDSCs (Table 1). Importantly, in the same experimental setting, we found that polyI:C treat-

ment induced none of the effects observed with VSSP inoculation.

The ability of VSSP to activate antigen-specific $CD8^+$ T cells was also tested in leukopenic mice. In this immunocompromised scenario, vaccination with a single dose of OVA/VSSP, at the time point corresponding to the lowest CD8 counts, induced significant antigen-specific CTL responses. In comparison, vaccination with three doses of OVA/polyI:C was not capable of inducing antigen-specific effector $CD8^+$ T cell activation. Furthermore, VSSP treatment of OVA/polyI:C vaccinated animals restored the dampened CTL responses in polyI:C-treated leukopenic mice, indicating that VSSP can function as an immunomodulator as well. This effect could be associated to the capacity of VSSP, different from polyI:C, to accelerate the recovery of effector $CD8^+$ memory T cells and to induce the expansion of DCs and less suppressive MDSCs.

Granulocyte colony-stimulating factor (G-CSF) is the standard growth factor used in the clinic to revert chemotherapy-induced leukopenia, but also has been reported to be a tumor-derived factor that induces MDSCs generation and recruitment[63]. Therefore we assessed whether treatment with recombinant G-CSF could restore the *in vivo* CTL response barely induced by OVA/polyI:C vaccine in CY-treated mice[62]. Administration of G-CSF has no impact in the impaired antigen-specific CTL response, possibly due to the expansion of MDSCs but also *via* G-CSF-induced Th2 responses[64] and the resulting differentiation of Tregs that may impair effector T lymphocyte proliferation[65]. However, when VSSP was given with G-CSF, the ability of VSSP to restore $CD8^+$ T cell function was not affected, which opens the possibility for their concomitant use in the clinic. Moreover, the functionality of MDSCs recruited in these experiments was additionally evaluated. As expected from previous reports, our data also demonstrated that, in leukopenic mice treated with G-CSF, the induced MDSCs were highly suppressive. Importantly, the concomitant treatment with VSSP dampened the inhibitory function of MDSCs expanded after G-CSF injection. To our knowledge, no other adjuvant has been tested in this immunosuppressive leukopenic scenario induced by chemotherapy.

ANTI-TUMOR ACTIVITY OF VSSP

Several pre-clinical studies support the anti-tumor efficacy of VSSP, whether used alone or in combination with other tumor-associated antigens different from the GM3 ganglioside. The combination of surgery and VSSP alone prevented tumor recurrence and improved survival in melanoma B16F10 tumor-bearing mice[66]. In a different tumor model, treatment of mice bearing MCA203 tumors with three doses of VSSP was sufficient to significantly delay tumor growth[42]. Of interest, GM3 ganglioside, an important component of VSSP, is highly expressed on both melanoma B16F10 and MCA203 sarcoma. Particularly in MCA203 tumor-bearing mice, treatment with VSSP alone caused a significant increase in the frequency of classical IFN-γ-producing $CD8^+$ T cells specific for MCA203 antigens, suggesting an antigen-spreading likely induced by the initial response against the GM3 ganglioside[42]. Moreover, VSSP-adjuvanted vaccines (both peptides and whole proteins) have shown anti-tumor activity. For instance, a vaccine containing the extracellular domain of murine epidermal growth factor receptor (EGFR) and VSSP has a potent anti-metastatic effect in the Lewis lung carcinoma model[67]. In a mouse model of cervical cancer induced by Human Papilloma Virus (HPV), the immunization with an E7-derived CTL peptide from HPV 16 mixed with VSSP induced regression of established tumors[68]. Therapeutic vaccination of EG.7 tumor-bearing mice with OVA or SIINFEKL peptide adjuvated in VSSP, but not SIINFEKL emulsified in IFA, caused a significant reduction of tumor growth[42]. However, VSSP administration alone to EL4 and C26GM tumor-bearing mice, with the same schedule associated with the inhibition of MDSCs suppressive function, does not delay tumor growth. One possible explanation for the absence of an anti-tumor effect of VSSP alone in these models is the lack of a tumor-associated antigen during treatment, and consequently, the absence of antigen-specific $CD8^+$ T cell activation. In fact, EL4 tumors express low levels of GM3 whereas an inappropriate exposure of this ganglioside on the surface of C26GM tumor cells has been observed[42]. Altogether, these data strongly suggest that the best induction of anti-tumor responses requires combining the abrogation of tumor-induced MDSCs with a specific stimulation of T lymphocytes, which can be successfully done by mixing a proper tumor-associated antigen with VSSP.

Finally, four therapeutic cancer vaccines employing VSSP as adjuvant are in clinical trials. An EGFR-based vaccine[67] is currently in Phase I clinical trials. A Phase I clinical trial in patients with advanced solid tumors using a formulation of a mutated vascular endothelial growth factor[69] and VSSP has been recently completed. In this trial, the most common adverse events were Grade 1 pain and erythema at injection site and Grade 1 fever [70]. Additionally, a gonadotropin releasing hormone-based vaccine[71] and a HPV-derived peptidic vaccine[72] are currently in Phase Ⅱ trials in prostate cancer patients and women with high-grade cervical intraepithelial neoplasia, respectively. Both vaccines have previously shown to be safe and immunogenic. The most frequent adverse event in patients receiving the HPV vaccine was local pain at the vaccination site, whereas fever, tremors and cramps were seen in few cases, but none exceeded Grade 1[72]. Another Phase I trial using VSSP alone in metastatic melanoma patients demonstrated the safety of this preparation even in the presence of Montanide ISA 51, with toxicity consisting of local reaction at the site of injection and mild fever and chills[49]. In this trial both humoral and cellular responses were induced by the VSSP treatment. Additionally, an ongoing physician-lead trial is evaluat-

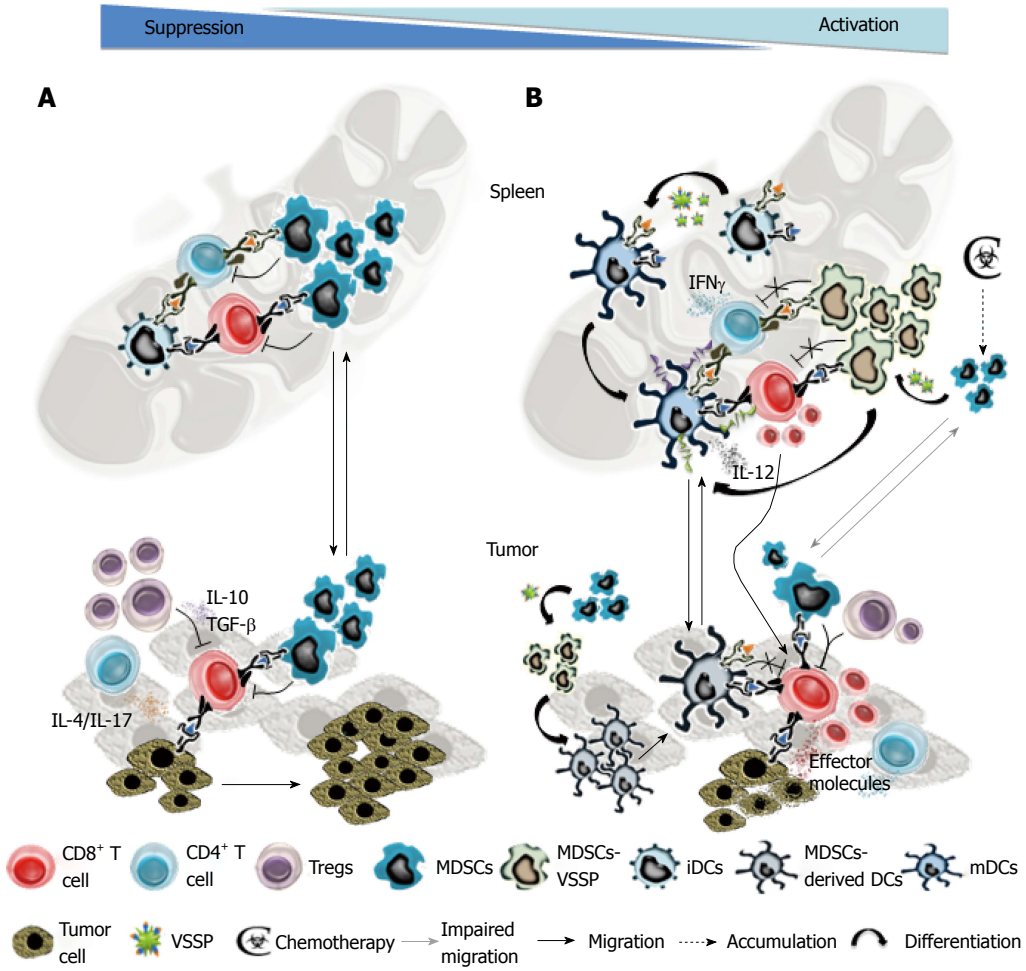


Figure 1 Schematic of potential immunomodulatory effects of very small size proteoliposomes in tumor-bearing hosts. A: Tumor-associated immunosuppressive networks prevent the elimination of neoplastic cells by specific T cells, thus contributing to tumor growth and metastasis; B: VSSP administration reduces the suppressive function of tumor-induced MDSCs, impairs their migration to the tumor microenvironment and promotes their differentiation towards DCs, both at the tumor and secondary lymphoid organs. VSSP also stimulates the activation and effector function of tumor-specific CTL, and combined with the concomitant reduction in the frequency of suppressive MDSCs and Tregs at the tumor site, further enhances elimination of neoplastic cells. An accelerated recovery from chemotherapy-induced leukopenia with VSSP treatment also contributes to a better anti-tumor response. VSSP: Very small size proteoliposomes; MDSCs: Myeloid-derived suppressor cells; DCs: Dendritic cells; CTL: Cytotoxic T lymphocytes; IL: Interleukin; TGF: Transforming growth factor.

ing the modulation of tumor-induced MDSCs by VSSP treatment alone in RCC patients.

CONCLUSION

The immunomodulatory and anti-tumor properties of VSSP are summarized in Figure 1. In tumor-bearing mice, activation and effector function of tumor-specific $CD8^+$ and $CD4^+$ T cells are impaired, among other factors, due to ineffective antigenic presentation by immature DCs and through multiple suppressive mechanisms exerted by MDSCs. Experimental evidence suggest that VSSP-based vaccines could promote cross-presentation of the formulated antigen by DCs, drive the full maturation of the DCs and, simultaneously, inhibit tumor-induced MDSCs immunosuppression. In addition, VSSP could induce Th1 polarization on tumor-specific $CD4^+$ T cells. All these effects may significantly enhance the proliferation and activation of tumor-specific $CD8^+$ T cells, thus eliciting robust anti-tumor immunity. VSSP also diminishes the migration of MDSCs towards the tumor site and promotes their differentiation into DCs. Tumor-infiltrating MDSCs have been related with the recruitment and expansion of Tregs[23,24,73], in addition to an impaired migration of effector T cells[74]. Thus, within the tumor microenvironment, VSSP treatment may tip the the balance between functional T cells vs suppressive MDSCs/Tregs to favor the immune effectors that ultimately lead to an anti-tumor response. The higher frequency of DCs could additionally contribute to activate T cells specific for other tumor antigens by capturing, processing and presenting the proteins released by dying tumor cells. In chemotherapy-treated individuals, VSSP also accelerates the homeostatic recovery of $CD8^+$ T cells and DCs, whereas the suppressive function of chemotherapy-induced MDSCs is abrogated. Altogether, these elements support the use of VSSP as a novel adjuvant or immunomodulator for active immunotherapy and, particularly, for the combination with chemotherapy in the clinical setting.

ACKNOWLEDGMENTS

We are grateful to Dr. Kelvin P (Roswell Park Cancer Institute, Buffalo, United States) for the language correction and critical review of the scientific content of this manuscript.

REFERENCES

1 **Kim R**, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. *Immunology* 2007; **121**: 1-14 [PMID: 17386080 DOI: 10.1111/j.1365-2567.2007.02587]
2 **Schreiber RD**, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011; **331**: 1565-1570 [PMID: 21436444 DOI: 10.1126/science.1203486]
3 **Gabrilovich DI**, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* 2012; **12**: 253-268 [PMID: 22437938 DOI: 10.1038/nri3175]
4 **Talmadge JE**, Gabrilovich DI. History of myeloid-derived suppressor cells. *Nat Rev Cancer* 2013; **13**: 739-752 [PMID: 24060865 DOI: 10.1038/nrc3581]
5 **Gallina G**, Dolcetti L, Serafini P, De Santo C, Marigo I, Colombo MP, Basso G, Brombacher F, Borrello I, Zanovello P, Bicciato S, Bronte V. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J Clin Invest* 2006; **116**: 2777-2790 [PMID: 17016559 DOI: 10.1172/JCI28828]
6 **Nagaraj S**, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, Herber DL, Schneck J, Gabrilovich DI. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* 2007; **13**: 828-835 [PMID: 17603493 DOI: 10.1038/nm1609]
7 **Liu C**, Yu S, Kappes J, Wang J, Grizzle WE, Zinn KR, Zhang HG. Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host. *Blood* 2007; **109**: 4336-4342 [PMID: 17244679 DOI: 10.1182/blood-2006-09-046201]
8 **Dolcetti L**, Peranzoni E, Ugel S, Marigo I, Fernandez Gomez A, Mesa C, Geilich M, Winkels G, Traggiai E, Casati A, Grassi F, Bronte V. Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. *Eur J Immunol* 2010; **40**: 22-35 [PMID: 19941314 DOI: 10.1002/eji.200939903]
9 **Movahedi K**, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, De Baetselier P, Van Ginderachter JA. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood* 2008; **111**: 4233-4244 [PMID: 18272812 DOI: 10.1182/blood-2007-07-099226]
10 **Peranzoni E**, Zilio S, Marigo I, Dolcetti L, Zanovello P, Mandruzzato S, Bronte V. Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol* 2010; **22**: 238-244 [PMID: 20171075 DOI: 10.1016/j.coi.2010.01.021]
11 **Youn JI**, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol* 2008; **181**: 5791-5802 [PMID: 18832739 DOI: 10.4049/jimmunol.181.8.5791]
12 **Corzo CA**, Cotter MJ, Cheng P, Cheng F, Kusmartsev S, Sotomayor E, Padhya T, McCaffrey TV, McCaffrey JC, Gabrilovich DI. Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells. *J Immunol* 2009; **182**: 5693-5701 [PMID: 19380816 DOI: 10.4049/jimmunol.0900092]
13 **Gabrilovich DI**, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009; **9**: 162-174 [PMID: 19197294 DOI: 10.1038/nri2506]
14 **Schmielau J**, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res* 2001; **61**: 4756-4760 [PMID: 11406548]
15 **Cuervo H**, Guerrero NA, Carbajosa S, Beschin A, De Baetselier P, Gironès N, Fresno M. Myeloid-derived suppressor cells infiltrate the heart in acute Trypanosoma cruzi infection. *J Immunol* 2011; **187**: 2656-2665 [PMID: 21804013 DOI: 10.4049/jimmunol.1002928]
16 **Lesokhin AM**, Hohl TM, Kitano S, Cortez C, Hirschhorn-Cymerman D, Avogadri F, Rizzuto GA, Lazarus JJ, Pamer EG, Houghton AN, Merghoub T, Wolchok JD. Monocytic CCR2(+) myeloid-derived suppressor cells promote immune escape by limiting activated CD8 T-cell infiltration into the tumor microenvironment. *Cancer Res* 2012; **72**: 876-886 [PMID: 22174368 DOI: 10.1158/0008-5472.CAN-11-1792]
17 **Nausch N**, Galani IE, Schlecker E, Cerwenka A. Mononuclear myeloid-derived "suppressor" cells express RAE-1 and activate natural killer cells. *Blood* 2008; **112**: 4080-4089 [PMID: 18753637 DOI: 10.1182/blood-2008-03-143776]
18 **Qin A**, Cai W, Pan T, Wu K, Yang Q, Wang N, Liu Y, Yan D, Hu F, Guo P, Chen X, Chen L, Zhang H, Tang X, Zhou J. Expansion of monocytic myeloid-derived suppressor cells dampens T cell function in HIV-1-seropositive individuals. *J Virol* 2013; **87**: 1477-1490 [PMID: 23152536 DOI: 10.1128/JVI.01759-12]
19 **Choi J**, Suh B, Ahn YO, Kim TM, Lee JO, Lee SH, Heo DS. CD15+/CD16low human granulocytes from terminal cancer patients: granulocytic myeloid-derived suppressor cells that have suppressive function. *Tumour Biol* 2012; **33**: 121-129 [PMID: 22081309 DOI: 10.1007/s13277-011-0254-6]
20 **Lechner MG**, Liebertz DJ, Epstein AL. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. *J Immunol* 2010; **185**: 2273-2284 [PMID: 20644162 DOI: 10.4049/jimmunol.1000901]
21 **Serafini P**. Myeloid derived suppressor cells in physiological and pathological conditions: the good, the bad, and the ugly. *Immunol Res* 2013; **57**: 172-184 [PMID: 24203443 DOI: 10.1007/s12026-013-8455-2]
22 **Bronte V**, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* 2005; **5**: 641-654 [PMID: 16056256 DOI: 10.1038/nri1668]
23 **Pan PY**, Ma G, Weber KJ, Ozao-Choy J, Wang G, Yin B, Divino CM, Chen SH. Immune stimulatory receptor CD40 is required for T-cell suppression and T regulatory cell activation mediated by myeloid-derived suppressor cells in cancer. *Cancer Res* 2010; **70**: 99-108 [PMID: 19996287 DOI: 10.1158/0008-5472.CAN-09-1882]
24 **Serafini P**, Mgebroff S, Noonan K, Borrello I. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res* 2008; **68**: 5439-5449 [PMID: 18593947 DOI: 10.1158/0008-5472.CAN-07-6621]
25 **Corzo CA**, Condamine T, Lu L, Cotter MJ, Youn JI, Cheng P, Cho HI, Celis E, Quiceno DG, Padhya T, McCaffrey TV, McCaffrey JC, Gabrilovich DI. HIF-1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *J Exp Med* 2010; **207**: 2439-2453 [PMID: 20876310 DOI: 10.1084/jem.20100587]
26 **Doedens AL**, Stockmann C, Rubinstein MP, Liao D, Zhang N, DeNardo DG, Coussens LM, Karin M, Goldrath AW, Johnson RS. Macrophage expression of hypoxia-inducible factor-1 alpha suppresses T-cell function and promotes tumor progression. *Cancer Res* 2010; **70**: 7465-7475 [PMID: 20841473 DOI: 10.1158/0008-5472.CAN-10-1439]
27 **Chioda M**, Peranzoni E, Desantis G, Papalini F, Falisi E, Solito S, Mandruzzato S, Bronte V. Myeloid cell diversification and complexity: an old concept with new turns in oncology. *Cancer Metastasis Rev* 2011; **30**: 27-43 [PMID: 21267772 DOI: 10.1007/s10555-011-9268-1]
28 **Yang L**, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis

B, Shyr Y, Matrisian LM, Carbone DP, Lin PC. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 2004; **6**: 409-421 [PMID: 15488763 DOI: 10.1016/j.ccr.2004.08.031]

29 **Du R**, Lu KV, Petritsch C, Liu P, Ganss R, Passegué E, Song H, Vandenberg S, Johnson RS, Werb Z, Bergers G. HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell* 2008; **13**: 206-220 [PMID: 18328425 DOI: 10.1016/j.ccr.2008.01.034]

30 **Pahler JC**, Tazzyman S, Erez N, Chen YY, Murdoch C, Nozawa H, Lewis CE, Hanahan D. Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response. *Neoplasia* 2008; **10**: 329-340 [PMID: 18392134]

31 **Yan HH**, Pickup M, Pang Y, Gorska AE, Li Z, Chytil A, Geng Y, Gray JW, Moses HL, Yang L. Gr-1+CD11b+ myeloid cells tip the balance of immune protection to tumor promotion in the premetastatic lung. *Cancer Res* 2010; **70**: 6139-6149 [PMID: 20631080 DOI: 10.1158/0008-5472.CAN-10-0706]

32 **Kusmartsev S**, Su Z, Heiser A, Dannull J, Eruslanov E, Kübler H, Yancey D, Dahm P, Vieweg J. Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. *Clin Cancer Res* 2008; **14**: 8270-8278 [PMID: 19088044 DOI: 10.1158/1078-0432.CCR-08-0165]

33 **Lathers DM**, Clark JI, Achille NJ, Young MR. Phase 1B study to improve immune responses in head and neck cancer patients using escalating doses of 25-hydroxyvitamin D3. *Cancer Immunol Immunother* 2004; **53**: 422-430 [PMID: 14648070 DOI: 10.1007/s00262-003-0459-7]

34 **Mirza N**, Fishman M, Fricke I, Dunn M, Neuger AM, Frost TJ, Lush RM, Antonia S, Gabrilovich DI. All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res* 2006; **66**: 9299-9307 [PMID: 16982775 DOI: 10.1158/0008-5472.CAN-06-1690]

35 **Ko JS**, Zea AH, Rini BI, Ireland JL, Elson P, Cohen P, Golshayan A, Rayman PA, Wood L, Garcia J, Dreicer R, Bukowski R, Finke JH. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. *Clin Cancer Res* 2009; **15**: 2148-2157 [PMID: 19276286 DOI: 10.1158/1078-0432.CCR-08-1332]

36 **Montero AJ**, Diaz-Montero CM, Kyriakopoulos CE, Bronte V, Mandruzzato S. Myeloid-derived suppressor cells in cancer patients: a clinical perspective. *J Immunother* 2012; **35**: 107-115 [PMID: 22306898 DOI: 10.1097/CJI.0b013e318242169f]

37 **Serafini P**, Meckel K, Kelso M, Noonan K, Califano J, Koch W, Dolcetti L, Bronte V, Borrello I. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. *J Exp Med* 2006; **203**: 2691-2702 [PMID: 17101732 DOI: 10.1084/jem.20061104]

38 **Crawford J**, Dale DC, Kuderer NM, Culakova E, Poniewierski MS, Wolff D, Lyman GH. Risk and timing of neutropenic events in adult cancer patients receiving chemotherapy: the results of a prospective nationwide study of oncology practice. *J Natl Compr Canc Netw* 2008; **6**: 109-118 [PMID: 18319047]

39 **Pardoll D**. Does the immune system see tumors as foreign or self? *Annu Rev Immunol* 2003; **21**: 807-839 [PMID: 12615893 DOI: 10.1146/annurev.immunol.21.120601.141135]

40 **Todryk S**. A sense of tumour for the immune system. *Immunology* 2002; **107**: 1-4 [PMID: 12225356 DOI: 10.1046/j.1365-2567.2002.01506.x]

41 **De Wilde V**, Van Rompaey N, Hill M, Lebrun JF, Lemaître P, Lhommé F, Kubjak C, Vokaer B, Oldenhove G, Charbonnier LM, Cuturi MC, Goldman M, Le Moine A. Endotoxin-induced myeloid-derived suppressor cells inhibit alloimmune responses via heme oxygenase-1. *Am J Transplant* 2009; **9**: 2034-2047 [PMID: 19681826 DOI: 10.1111/j.1600-6143.2009.02757]

42 **Fernández A**, Mesa C, Marigo I, Dolcetti L, Clavell M, Oliver L, Fernández LE, Bronte V. Inhibition of tumor-induced myeloid-derived suppressor cell function by a nanoparticulated adjuvant. *J Immunol* 2011; **186**: 264-274 [PMID: 21135171 DOI: 10.4049/jimmunol.1001465]

43 **Ohkusu-Tsukada K**, Ohta S, Kawakami Y, Toda M. Adjuvant effects of formalin-inactivated HSV through activation of dendritic cells and inactivation of myeloid-derived suppressor cells in cancer immunotherapy. *Int J Cancer* 2011; **128**: 119-131 [PMID: 20232389 DOI: 10.1002/ijc.25319]

44 **Shirota Y**, Shirota H, Klinman DM. Intratumoral injection of CpG oligonucleotides induces the differentiation and reduces the immunosuppressive activity of myeloid-derived suppressor cells. *J Immunol* 2012; **188**: 1592-1599 [PMID: 22231700 DOI: 10.4049/jimmunol.1101304]

45 **Wang Z**, Jiang J, Li Z, Zhang J, Wang H, Qin Z. A myeloid cell population induced by Freund adjuvant suppresses T-cell-mediated antitumor immunity. *J Immunother* 2010; **33**: 167-177 [PMID: 20145547 DOI: 10.1097/CJI.0b013e3181bed2ba]

46 **Estevez F**, Carr A, Solorzano L, Valiente O, Mesa C, Barroso O, Sierra GV, Fernandez LE. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). *Vaccine* 1999; **18**: 190-197 [PMID: 10501249 DOI: 10.1016/S0264-410X(99)00219-4]

47 **Mesa C**, De León J, Rigley K, Fernández LE. Very small size proteoliposomes derived from Neisseria meningitidis: an effective adjuvant for Th1 induction and dendritic cell activation. *Vaccine* 2004; **22**: 3045-3052 [PMID: 15297054 DOI: 10.1016/j.vaccine.2004.02.010]

48 **Venier C**, Guthmann MD, Fernández LE, Fainboim L. Innate-immunity cytokines induced by very small size proteoliposomes, a Neisseria-derived immunological adjuvant. *Clin Exp Immunol* 2007; **147**: 379-388 [PMID: 17223981 DOI: 10.1111/j.1365-2249.2006.03297.x]

49 **Guthmann MD**, Bitton RJ, Carnero AJ, Gabri MR, Cinat G, Koliren L, Lewi D, Fernandez LE, Alonso DF, Gómez DE, Fainboim L. Active specific immunotherapy of melanoma with a GM3 ganglioside-based vaccine: a report on safety and immunogenicity. *J Immunother* 2004; **27**: 442-451 [PMID: 15534488 DOI: 10.1097/00002371-200411000-00004]

50 **Mesa C**, de León J, Fernández LE. Very small size proteoliposomes derived from Neisseria meningitidis: An effective adjuvant for generation of CTL responses to peptide and protein antigens. *Vaccine* 2006; **24**: 2692-2699 [PMID: 16316710 DOI: 10.1016/j.vaccine.2005.08.111]

51 **Parmiani G**, Castelli C, Pilla L, Santinami M, Colombo MP, Rivoltini L. Opposite immune functions of GM-CSF administered as vaccine adjuvant in cancer patients. *Ann Oncol* 2007; **18**: 226-232 [PMID: 17116643 DOI: 10.1093/annonc/mdl158]

52 **Serafini P**, Carbley R, Noonan KA, Tan G, Bronte V, Borrello I. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res* 2004; **64**: 6337-6343 [PMID: 15342423 DOI: 10.1158/0008-5472.CAN-04-0757]

53 **Morecki S**, Gelfand Y, Yacovlev E, Eizik O, Shabat Y, Slavin S. CpG-induced myeloid CD11b+Gr-1+ cells efficiently suppress T cell-mediated immunoreactivity and graft-versus-host disease in a murine model of allogeneic cell therapy. *Biol Blood Marrow Transplant* 2008; **14**: 973-984 [PMID: 18721760 DOI: 10.1016/j.bbmt.2008.06.018]

54 **Martino A**, Badell E, Abadie V, Balloy V, Chignard M, Mistou MY, Combadière B, Combadière C, Winter N. Mycobacterium bovis bacillus Calmette-Guérin vaccination mobilizes innate myeloid-derived suppressor cells restraining in vivo T cell priming via IL-1R-dependent nitric oxide production. *J Immunol* 2010; **184**: 2038-2047 [PMID: 20083674 DOI: 10.4049/jimmunol.0903348]

55 **Cauley LS**, Miller EE, Yen M, Swain SL. Superantigen-induced CD4 T cell tolerance mediated by myeloid cells and

IFN-gamma. *J Immunol* 2000; **165**: 6056-6066 [PMID: 11086037 DOI: 10.4049/jimmunol.165.11.6056]
56 **Scumpia PO**, Kelly-Scumpia KM, Delano MJ, Weinstein JS, Cuenca AG, Al-Quran S, Bovio I, Akira S, Kumagai Y, Moldawer LL. Cutting edge: bacterial infection induces hematopoietic stem and progenitor cell expansion in the absence of TLR signaling. *J Immunol* 2010; **184**: 2247-2251 [PMID: 20130216 DOI: 10.4049/jimmunol.0903652]
57 **Murphey ED**, Lin CY, McGuire RW, Toliver-Kinsky T, Herndon DN, Sherwood ER. Diminished bacterial clearance is associated with decreased IL-12 and interferon-gamma production but a sustained proinflammatory response in a murine model of postseptic immunosuppression. *Shock* 2004; **21**: 415-425 [PMID: 15087817 DOI: 10.1097/00024382-200405000-00004]
58 **Makarenkova VP**, Bansal V, Matta BM, Perez LA, Ochoa JB. CD11b+/Gr-1+ myeloid suppressor cells cause T cell dysfunction after traumatic stress. *J Immunol* 2006; **176**: 2085-2094 [PMID: 16455964 DOI: 10.4049/jimmunol.176.4.2085]
59 **Fernández A**, Oliver L, Alvarez R, Hernández A, Raymond J, Fernández LE, Mesa C. Very small size proteoliposomes abrogate cross-presentation of tumor antigens by myeloid-derived suppressor cells and induce their differentiation to dendritic cells. *J Immunother Cancer* 2014; **2**: 5 [PMID: 24829762 DOI: 10.1186/2051-1426-2-5]
60 **Greifenberg V**, Ribechini E, Rössner S, Lutz MB. Myeloid-derived suppressor cell activation by combined LPS and IFN-gamma treatment impairs DC development. *Eur J Immunol* 2009; **39**: 2865-2876 [PMID: 19637228 DOI: 10.1002/eji.200939486]
61 **Källberg E**, Vogl T, Liberg D, Olsson A, Björk P, Wikström P, Bergh A, Roth J, Ivars F, Leanderson T. S100A9 interaction with TLR4 promotes tumor growth. *PLoS One* 2012; **7**: e34207 [PMID: 22470535 DOI: 10.1371/journal.pone.0034207]
62 **Oliver L**, Fernández A, Raymond J, López-Requena A, Fernández LE, Mesa C. Very small size proteoliposomes derived from Neisseria meningitidis: an effective adjuvant for antigen-specific cytotoxic T lymphocyte response stimulation under leukopenic conditions. *Vaccine* 2012; **30**: 2963-2972 [PMID: 22391399 DOI: 10.1016/j.vaccine.2012.02.054]
63 **Waight JD**, Hu Q, Miller A, Liu S, Abrams SI. Tumor-derived G-CSF facilitates neoplastic growth through a granulocytic myeloid-derived suppressor cell-dependent mechanism. *PLoS One* 2011; **6**: e27690 [PMID: 22110722 DOI: 10.1371/journal.pone.0027690]
64 **Pan L**, Delmonte J, Jalonen CK, Ferrara JL. Pretreatment of donor mice with granulocyte colony-stimulating factor polarizes donor T lymphocytes toward type-2 cytokine production and reduces severity of experimental graft-versus-host disease. *Blood* 1995; **86**: 4422-4429 [PMID: 8541530]
65 **Rutella S**, Pierelli L, Bonanno G, Sica S, Ameglio F, Capoluongo E, Mariotti A, Scambia G, d'Onofrio G, Leone G. Role for granulocyte colony-stimulating factor in the generation of human T regulatory type 1 cells. *Blood* 2002; **100**: 2562-2571 [PMID: 12239170 DOI: 10.1182/blood-2001-12-0291]
66 **Gabri MR**, Mazorra Z, Ripoll GV, Mesa C, Fernandez LE, Gomez DE, Alonso DF. Complete antitumor protection by perioperative immunization with GM3/VSSP vaccine in a preclinical mouse melanoma model. *Clin Cancer Res* 2006; **12**: 7092-7098 [PMID: 17145833 DOI: 10.1158/1078-0432.CCR-06-1075]
67 **Ramírez BS**, Pestana ES, Hidalgo GG, García TH, Rodríguez RP, Ullrich A, Férnandez LE. Active antimetastatic immunotherapy in Lewis lung carcinoma with self EGFR extracellular domain protein in VSSP adjuvant. *Int J Cancer* 2006; **119**: 2190-2199 [PMID: 16841332 DOI: 10.1002/ijc.22085]
68 **Torréns I**, Mendoza O, Batte A, Reyes O, Fernández LE, Mesa C, Guillén G. Immunotherapy with CTL peptide and VSSP eradicated established human papillomavirus (HPV) type 16 E7-expressing tumors. *Vaccine* 2005; **23**: 5768-5774 [PMID: 16112257 DOI: 10.1016/j.vaccine.2005.07.049]
69 **Bequet-Romero M**, Morera Y, Ayala-Ávila M, Ancizar J, Soria Y, Blanco A, Suárez-Alba J, Gavilondo JV. CIGB-247: a VEGF-based therapeutic vaccine that reduces experimental and spontaneous lung metastasis of C57Bl/6 and BALB/c mouse tumors. *Vaccine* 2012; **30**: 1790-1799 [PMID: 22240345 DOI: 10.1016/j.vaccine.2012.01.006]
70 **Gavilondo JV**, Hernández-Bernal F, Ayala-Ávila M, de la Torre AV, de la Torre J, Morera-Díaz Y, Bequet-Romero M, Sánchez J, Valenzuela CM, Martín Y, Selman-Housein KH, Garabito A, Lazo OC. Specific active immunotherapy with a VEGF vaccine in patients with advanced solid tumors. results of the CENTAURO antigen dose escalation phase I clinical trial. *Vaccine* 2014; **32**: 2241-2250 [PMID: 24530151 DOI: 10.1016/j.vaccine.2013.11.102]
71 **Aguilar FF**, Barranco JJ, Fuentes EB, Aguilera LC, Sáez YL, Santana MD, Vázquez EP, Baker RB, Acosta OR, Pérez HG, Nieto GG. Very small size proteoliposomes (VSSP) and Montanide combination enhance the humoral immuno response in a GnRH based vaccine directed to prostate cancer. *Vaccine* 2012; **30**: 6595-6599 [PMID: 22921738 DOI: 10.1016/j.vaccine.2012.08.020]
72 **Solares AM**, Baladron I, Ramos T, Valenzuela C, Borbon Z, Fanjull S, Gonzalez L, Castillo D, Esmir J, Granadillo M, Batte A, Cintado A, Ale M, Fernandez de Cossio ME, Ferrer A, Torrens I, Lopez-Saura P. Safety and Immunogenicity of a Human Papillomavirus Peptide Vaccine (CIGB-228) in Women with High-Grade Cervical Intraepithelial Neoplasia: First-in-Human, Proof-of-Concept Trial. *ISRN Obstet Gynecol* 2011; **2011**: 292951 [PMID: 21748025 DOI: 10.5402/2011/292951]
73 **Schlecker E**, Stojanovic A, Eisen C, Quack C, Falk CS, Umansky V, Cerwenka A. Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth. *J Immunol* 2012; **189**: 5602-5611 [PMID: 23152559 DOI: 10.4049/jimmunol.1201018]
74 **Molon B**, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, De Palma A, Mauri P, Monegal A, Rescigno M, Savino B, Colombo P, Jonjic N, Pecanic S, Lazzarato L, Fruttero R, Gasco A, Bronte V, Viola A. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *J Exp Med* 2011; **208**: 1949-1962 [PMID: 21930770 DOI: 10.1084/jem.20101956]

P- Reviewer: Fukuda S, Magnusson LU **S- Editor**: Song XX
L- Editor: A **E- Editor**: Wang CH

World Journal of Immunology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5411/wji.v4.i2.107

World J Immunol 2014 July 27; 4(2): 107-115
ISSN 2219-2824 (online)

REVIEW

Role of host immune responses in sequence variability of HIV-1 Vpu

Zafrul Hasan, Doreen Kamori, Takamasa Ueno

Zafrul Hasan, Doreen Kamori, Takamasa Ueno, Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan
Takamasa Ueno, International Research Center for Medical Sciences (IRCMS), Kumamoto University, Kumamoto 860-0811, Japan
Author contributions: Hasan Z and Kamori D contributed equally to this work; Hasan Z and Kamori D generated the figures and wrote the manuscript; Ueno T designed the study and contributed to the writing of the manuscript.
Supported by A Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture (MEXT) of Japan; and A Grant-in-Aid for AIDS Research from the Ministry of Health, Labor, and Welfare of Japan; The Scholarship for the International Priority Graduate Programs, to Hasan Z and Kamori D; Advanced Graduate Courses for International Students (Doctoral Course), MEXT, Japan, to Hasan Z and Kamori D
Correspondence to: Takamasa Ueno, PhD, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. uenotaka@kumamoto-u.ac.jp
Telephone: +81-96-3736826 Fax: +81-96-3736825
Received: March 7, 2014 Revised: April 19, 2014
Accepted: June 14, 2014
Published online: July 27, 2014

Abstract

Viral protein U (Vpu) is an accessory protein associated with two main functions important in human immunodeficiency virus type 1 (HIV-1) replication and dissemination; these are down-regulation of CD4 receptor through mediating its proteasomal degradation and enhancement of virion release by antagonizing tetherin/BST2. It is also well established that Vpu is one of the most highly variable proteins in the HIV-1 proteome. However it is still unclear what drives Vpu sequence variability, whether Vpu acquires polymorphisms as a means of immune escape, functional advantage, or otherwise. It is assumed that the host-pathogen interaction is a cause of polymorphic phenotype of Vpu and that the resulting functional heterogeneity of Vpu may have critical significance *in vivo*. In order to comprehensively understand Vpu variability, it is important to integrate at the population level the genetic association approaches to identify specific amino acid residues and the immune escape kinetics which may impose Vpu functional constraints *in vivo*. This review will focus on HIV-1 accessory protein Vpu in the context of its sequence variability at population level and also bring forward evidence on the role of the host immune responses in driving Vpu sequence variability; we will also highlight the recent findings that illustrate Vpu functional implication in HIV-1 pathogenesis.

Key words: Human immunodeficiency virus type 1; Vpu; Sequence variability; Immune responses; Human leukocyte antigen class I

Core tip: Viral protein U (Vpu) is a highly polymorphic human immunodeficiency virus type 1 (HIV-1) accessory protein; however factors that are attributable to Vpu sequence variability are not well defined. In this review we have focused on the immune responses both innate (natural killer cells) and adaptive (cellular and humoral) immunity that are directed towards HIV-1 Vpu and we also show the interaction between Vpu and host cellular factors. We also highlight evidence that suggests interaction between the host immune responses and Vpu may contribute to Vpu sequence variability. Finally we have summarized the current knowledge on HIV-1 Vpu functions including Vpu evasion activities from the host immune surveillance.

Hasan Z, Kamori D, Ueno T. Role of host immune responses in sequence variability of HIV-1 Vpu. *World J Immunol* 2014; 4(2): 107-115 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i2/107.htm DOI: http://dx.doi.org/10.5411/wji.v4.i2.107

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) demonstrates a significant genetic diversity due to its high

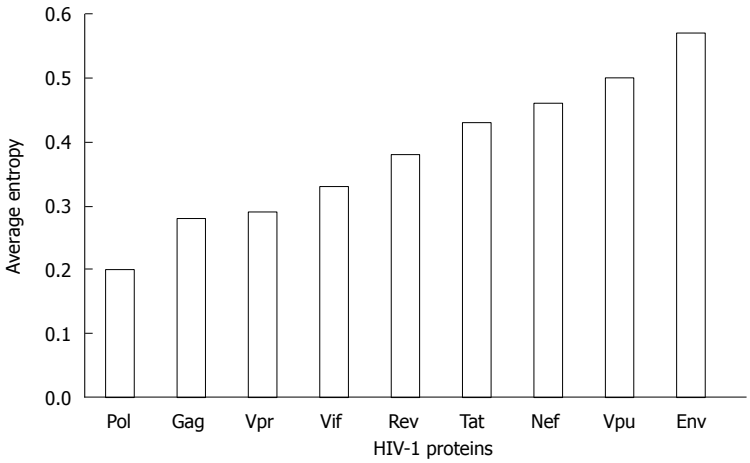


Figure 1 Sequence variability of human immunodeficiency virus type 1 proteins. The sequence variability of nine proteins of human immunodeficiency virus type 1 (HIV-1) shown in the graph was determined by using Shannon entropy approach[24,90]. The full genome clade B sequences of the individual patients were retrieved from Los Alamos database (n = 544). Vpu: Viral protein U.

mutation rate; so far this extraordinary diversity has been a major setback in development of vaccine and antiretroviral drugs. Low fidelity of reverse transcriptase that give rise to error prone replication process, high progeny production, turnover rates and recombination of circulating HIV-1 strains are some of the viral factors that contributes to HIV-1 diversity[1-3]. The adaptive potential of HIV-1 is shaped by both virus and the host immune factors, in other words both the diversifying and purifying selection factors influence HIV-1 diversity. In fact, strong evidence has also indicated that the host immune responses influence HIV-1 diversity by selection of escape mutations[4-6]. Thus a comprehensive analysis of the dynamics of polymorphisms in HIV-1 proteins is a powerful tool to reveal actual interactions between HIV-1 and the host immune system[7-9].

HIV-1 viral protein U (Vpu) is a 16-kDa accessory protein[10] responsible for various functions such as CD4 down-regulation[11-13] and enhancement of virion release by antagonizing tetherin/BST2[14-17]. Interestingly, functionally competent Vpu (with respect to BST-2 antagonistic activity) were only found in the pandemic group M subtypes, suggesting that Vpu functional adaptation may confer pandemic spread of this HIV-1 subtype[18]. In general, the host genetic factor is one of the main driving force of sequence polymorphism in HIV-1[18], as evidenced in HIV-1 Nef[17,19-21] and Env[22,23] proteins whose highly polymorphic phenotype is mostly attributed by the host immune responses such as HLA class I-restricted CD8+ T lymphocytes and neutralizing antibodies, respectively. However, it is still unclear to what extent the host immune responses influence Vpu sequence variation. This review focuses on the role of host immune responses in Vpu sequence variability. Briefly, we also discuss the current understanding of Vpu functions including evasion of the immune system and their implication in viral pathogenesis.

SEQUENCE VARIABILITY OF VPU

Vpu exhibit a stable reading frame *in vivo* despite being a highly variable protein, suggesting functional importance of Vpu in HIV-1 replication and persistence. Furthermore, it has evidently been shown that only HIV-1 strains of the pandemic M group evolved a fully functional Vpu that efficiently antagonizes human tetherin/BST-2; this suggests that Vpu evolutional adaptation may be associated with the pandemic spread of HIV-1[18]. Several studies have demonstrated the extent of Vpu sequence variability both at inter- and intra-patient level. By using the 101 aligned amino acid sequences of entire HIV-1 genome, one study showed that Vpu had the highest average entropy score in comparison to other proteins in HIV-1 genome[24]. Another study analyzing the intra-patient diversity and adaptation of non-structural genes in primary HIV-1 subtype C infection reported that *vpu* compared to *vif, vpr, tat exon 1* and *rev exon 1* genes has the highest mean of intra-patient diversity that increased gradually[25]. We retrieved full lengths clade B sequences (n = 544) of HIV-1 proteins (Gag, Pol, Env, Nef, Vif, Vpu, Vpr, Tat and Rev) from Los Alamos database and the average entropy score of each protein was determined. Vpu was observed to be one of the proteins with the highest average entropy score (Figure 1), confirming the highly variable nature of Vpu at population level. However, interestingly, a recent study has shown that despite extensive Vpu sequence variation in HIV-1 infected individuals, Vpu functions (CD4 cell surface downregulation and tetherin counteraction activity) were maintained[26].

IMMUNE RESPONSES TOWARDS VPU

Humoral immunity

Several studies have reported Vpu-specific humoral immune responses during HIV-1 infection[27-31]. However there has been some controversy on correlation between the presence of anti-Vpu Ab responses in HIV-infected patients' sera and clinical outcome. Some studies have indicated that anti-Vpu Ab responses may influence the clinical outcomes in HIV-1 infected individuals[27,28,30,31]; while on the other hand other studies have showed no correlation[29]. These findings indicate that Vpu is indeed a target of antibodies although no evidence yet support that such antibody responses influence the Vpu variability. The epitopic regions for such antibodies reported include 37-50[30] and 68-81[28] of Vpu; nonetheless there

is no specific Vpu activity mapped to these regions so far. However, considering that Vpu is a small protein (81 amino acids); it is intriguing to test whether such Vpu-specific antibodies can inhibit Vpu functions and subvert viral replication.

Cellular immunity

A growing number of clinical evidence has suggested that HLA-restricted, HIV-specific CD8+ cytotoxic T lymphocytes (CTL) is mainly involved in controlling HIV-1 replication[32-34]. CTL responses have been well appreciated in SIV-infected macaque's model[32,33] and in HIV-1 infected patients of both acute[35,36] and chronic[37] phases as well as in elite controllers who spontaneously suppress viral replication below detection limit[38,39]. HLA-restricted CTL responses are thought to be the main driving force of HIV-1 control and viral evolution[40-43]. The viral polymorphism in response to immune selective pressures follows predictable patterns and kinetics at the population and these immune "footprints/landscape" could be predictable based on the autologous viral sequences and the host immune genetics[9,42,44]. However, Vpu has been reported to be a poor target for CD8+ T cells as revealed by interferon (IFN)-γ Elispot assay[45], because only some few epitopes were identified and less than 3% of patients showed detectable Vpu-specific CD8+ T cell responses. Although several HLA-restricted CTL epitopes of Vpu are reported[45-49], this protein is less targeted by CTLs at least compared to the Nef protein. Consistently, our previous study showed only three HLA-associated polymorphisms in Vpu at Glu-5 with HLA-C*03 and Arg-37, Lsy-37 with HLA-A*3303 in a chronic HIV-infected patient cohort in Japan (n = 216), indicating that the HLA class I has minor contribution (2% of the total codons) towards Vpu variability[50]. The increased numbers of subjects to 516 showed similar results (DK, ZH, and TU: unpublished observation). Furthermore, an international large IHAC cohort (International HIV Adaptation Collaborative, n = 1888) identified that only 26.3% of the highly variable Vpu codons exhibited statistically significant HLA class I associations[20]. Although the HLA class I -associated viral polymorphisms observed in the two cohorts suggested to be influenced by several factors such as the host genetic profiles, mixture of multiethnic populations, studied sample size, geographical location and circulating HIV-strains, these results suggest that HLA-associated polymorphisms are only partly attributable to the Vpu variability (Figure 2). However, it is of note that the low CTL responses observed in the previous studies[45,51] and subtle numbers of HLA-associated polymorphisms[20,50] may be an underestimation due to the current technical limitation toward a highly variable protein, even though a number of studies reported a plenty of CTL targeting[52,53] and HLA-associated polymorphisms in Nef[19,20,42], which showed comparable variability with Vpu at a population level (Figure 1).

Natural killer cells

A number of evidence suggests that natural killer (NK) cells have an important role in control of HIV-1 infection[54-56]. Assuming that NK cells may act as a selective force, as similar to CTLs, HIV-1 may leave footprints as viral polymorphisms in association with polymorphic NK cell ligand such as killer-cell immunoglobulin-like receptors (KIR). In fact, one study identified 22 amino-acid polymorphisms within the HIV-1 clade B sequence that are significantly associated with the expression of specific KIR genes in chronically HIV-1 infected, treatment naïve patients (n =91)[44]. Three (13.6%) of these KIR associated polymorphisms were located in Vpu at positions Ser-3 and Vpu-Env overlapping region (Met-71 and His-74) (Figure 2)[44]. In addition, the HIV-1-specific antibody-dependent NK cell cytotoxicity is identified towards a 13-mer Vpu peptide (^{69}EMGHHAPWDVD-DL^{81})[57]. Such responses are also observed toward Env[58] and Nef[59] in HIV-1 infected patients as well. However, there is no evidence at the moment that show antibody-dependent NK cell cytotoxicity associates with viral polymorphisms.

VPU FUNCTIONALITY INCLUDING IMMUNE EVASION ACTIVITY

In order to conquer the hostile host environment, viruses need to evolve and develop critical interactions with the host cellular factors. Vpu does not only play important role in HIV-1 pathogenesis through CD4 receptor degradation[11] and enhancement of virion release from infected cells by antagonizing tetherin/BST-2[60-62]; but Vpu has also evolved to interact with and modulate other host surface receptors and factors (Figure 3).

Vpu induces CD4 receptor degradation

Vpu induces the rapid degradation of newly synthesized CD4 receptor molecules that are retained together with Env precursor protein (gp160) in the endoplasmic reticulum[13]. The cytoplasmic domain of Vpu and the DSGxxS motif are critical in interaction with and degradation of CD4, respectively[12,63] (Figure 2). The degradation process is achieved by Vpu recruiting β-TrCP and then interacts with CD4 cytoplasmic domain and subsequently subject CD4 to degradation by the ubiquitin-proteasome pathway[11,64]. In doing so Vpu contributes to the suppression of HIV-1 primary receptor at the surface of the infected cell.

Vpu enhances virion release

Enhancement of virion release by Vpu has been shown to be achieved through antagonizing tetherin/BST-2, an IFN regulated host restriction factor. BST-2 directly binds to virions and hence retains them on the surface of infected cells[61,62]. Vpu through AxxxAxxxA motif in transmembrane domain directly interacts with BST-2 transmembrane domain, the Vpu DSGxxS and [D/E]XXXL[L/I/V] motifs in the cytoplasmic domain also play crucial role in ensuring BST-2 downmodulation[15,65,66] (Figure 2). Previous studies indicated BST-2

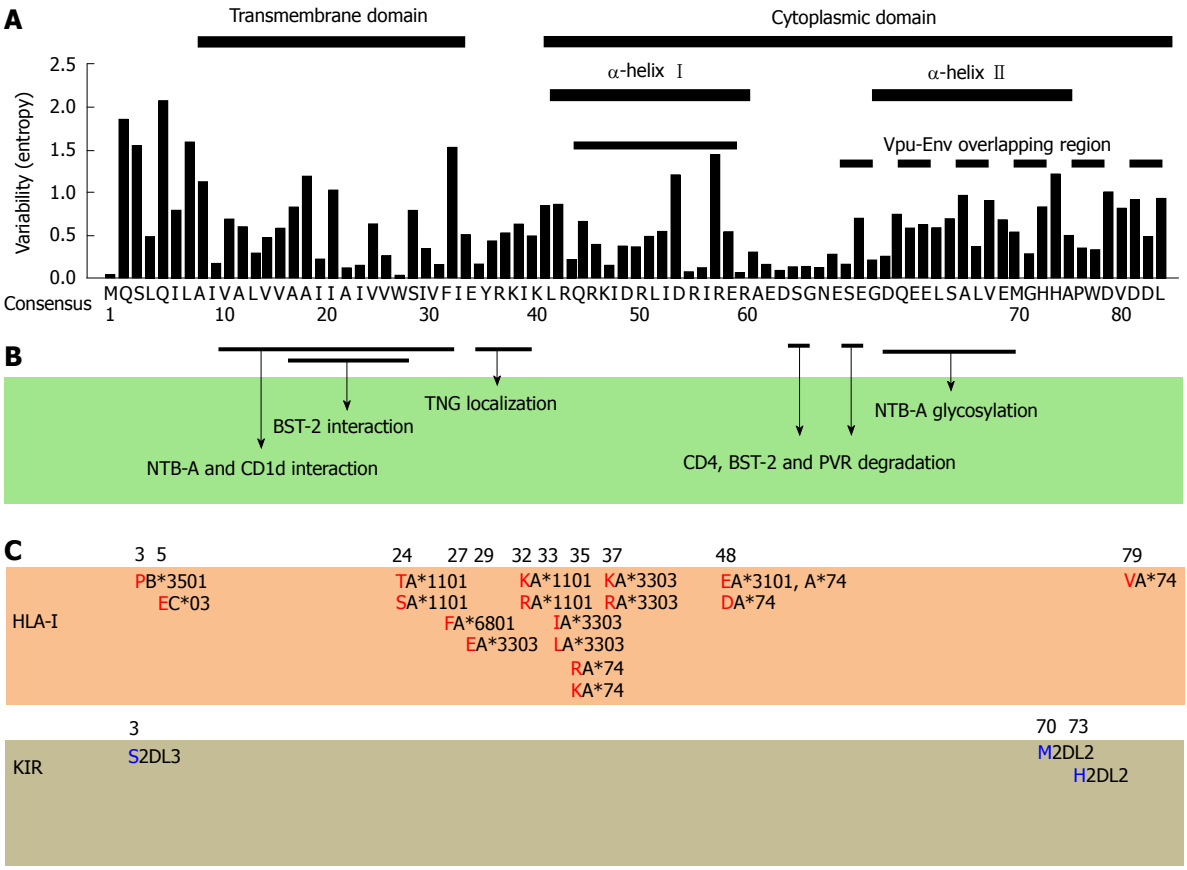


Figure 2 Correlation among amino-acid codon variability, functional regions, and host-mediated immune escape map of human immunodeficiency virus type 1 viral protein U. A: Amino acid codon variability is assigned to each position of Vpu using Shannon entropy approach[24,90]. Sequences were retrieved from Los Alamos database (*n* =1139), the consensus subtype B sequence is indicated as a reference; B: Interacting positions and domains responsible for the indicated functions of human immunodeficiency virus type 1 (HIV-1) Vpu are shown[14,62,91]; C: Immune escape map shows amino acid codons and residues (red and blue) associated with HLA-I alleles[20,45,50] and killer-cell immunoglobulin-like receptors (KIR)[44], respectively. The specific alleles are indicated in black adjacent to the amino acid.

downmodulation is through β-TrCP-dependent proteasomal degradation pathway[67] while others suggested the β-TrCP-dependent endo-lysosomal pathway[65,68]. In contrast, recent studies showed that BST-2 antagonistic activity by Vpu takes place in the trans-Golgi networks (TGN)[14]. Vpu interferes with anterograde transport of BST-2 to the cell surface subsequently leading to BST-2 trapping in the TGN[15-17,69].

Vpu modulation of other cell surface receptors and host factors

Recent studies have indicated that Vpu is emerging as a viral factor with a range of activities devoted to counteracting host innate and adaptive immunity including the modulation of NK cell co-activation ligand NK-T and B cell antigen (NTB-A)[70], PVR activating ligand of NK cells[71], and CD1d[72,73] (Figure 3).

NTB-A triggering is necessary for induction of efficient lysis of target cells upon engagement of the activating receptor NKG2D[74]. The Ser-52 and Ser-56 residues important for CD4 and BST-2 degradation did not affect NTB-A expression, indicating that the down modulation of NTB-A by Vpu is mediated by different domains[70]. A recent study has shown that downmodulation of NTB-A is achieved by Vpu interfering with the anterograde transport of NTB-A by retaining it within the Golgi compartment and hence affects its glycosylation pattern that subsequently reduces surface expression of NTB-A[75].

PVR (CD155, Necl-5) is a ligand for the activating receptor DNAM-1 (CD226) expressed by NK cells[76,77]. PVR downmodulation by Nef and Vpu is another strategy evolved by HIV-1 to avoid NK cell-mediated lysis of infected cells[71]. PVR downregulation alters multiple important PVR-mediated innate cellular immune processes such as adhesion and migration, and therefore may influence HIV-1 pathogenesis.

CD1d molecules are important in dendritic cells for lipid antigen presentation to CD1d-restricted NKT cells[78,79]. CD1d and CD1d-restricted NKT cells are present at pathogen entry sites thus play a crucial role in early immune responses[80]. Vpu has been shown to be the major viral factor that inhibit recycling of CD1d from the endosomal compartment back to cell surface through retaining CD1d in early endosomes[72].

Vpu has also been implicated in inhibition of ubiquitination and degradation of p53 (a substrate of $SCF^{\beta\text{-}TrCP}$ ligase complex). The successful interaction of $SCF^{\beta\text{-}TrCP}$ complex with β-TrCP binding motif ($DS_{52}GNES_{56}$) present in Vpu has been shown to be essential[81]. It was observed that Vpu mutants with alanine substitutions

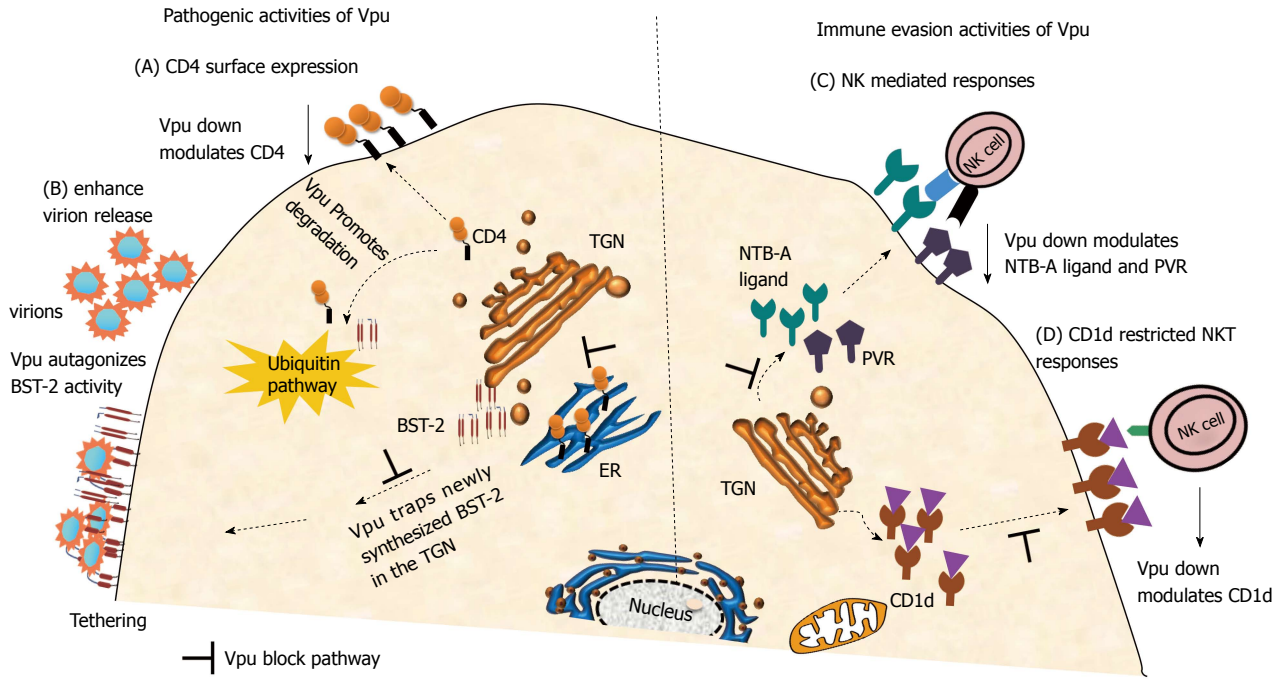


Figure 3 Viral protein U functionality including immune evasion activity. The schematic representation of the cell illustrates some key functions of viral protein U (Vpu) including immune evasion activities. (A): Panel A illustrates CD4 down regulation by Vpu through degradation in a β-TrCP dependent ubiquitination pathway[11,12,64]; (B): Panel B demonstrates enhancement of virion release by Vpu through antagonizing BST-2, which is achieved through direct interaction with BST-2 which subsequently leads to trapping of BST-2 in the trans-Golgi networks[14-16] and also indicates β-TrCP dependent ubiquitination of BST-2[62,65,66]; (C): Panel C demonstrates Vpu evasion of NK cell recognition through down modulation of NTB-A ligand[70] and PVR[71]; D: Panel D shows down modulation of CD1d from cell surface hence avoid CD1d-restricted NKT cell responses[72,73]. NK: Natural killer; NKT: Natural killer T.

(DA$_{52}$GNEA$_{56}$) failed to stabilize p53 and did not prevent its ubiquitination. This suggested that Vpu is able to achieve modulation of p53 through competing efficiently with p53 protein for the β-TrCP subunit of the SCF complex and hence inhibits subsequent ubiquitination of p53 protein. The modulation of p53 positively correlated with apoptosis during the late stages of HIV-1 infection[81].

Finally, although Vpu showed multiple functions *in vitro* and *ex vivo*, it is yet clear how and what functions of Vpu are important in viral pathogenesis *in vivo*.

CONCLUSION

The current knowledge on factors that are attributed to Vpu polymorphism has not been quite sufficient; therefore this prompt for further analysis to reveal the unresolved questions of why Vpu is so variable and what factors drive Vpu polymorphism. In order to define the complex dynamics of HIV-1 Vpu evolution, immune escape patterns, and functional adaptation during the course of infection, further insight is needed on the role of host genetics and other immune selection pressures towards shaping HIV-1 Vpu diversity. The emergence of advanced DNA sequencing technologies such as ultra-deep sequence which is superior and more sensitive than Sanger sequence methods has made it possible to accurately detect and analyze minor variants of HIV-1 within a host[82-85]. Furthermore, the establishment of different contemporaneous cohorts of HIV-1-infected individuals worldwide enables us to examine to what extent the host immune components play a role on viral adaptation and/or evolution at both intra- and inter-patients' level.

So far the current studies have indicated that the host immune responses directed towards Vpu is not entirely attributable to HIV-1 Vpu variation (Figure 2), it is therefore crucial to apprehend other factors that may explain Vpu variation. Of note previous studies have identified immune responses directed towards Vpu, using peptides of HIV-1 consensus sequences[45,57]. However, ironically due to Vpu polymorphic nature itself, these results may mask the exact extent to which immune responses contribute to Vpu sequence variation. Alternatively, HIV-1 like other RNA viruses has evolved to shorten its genome length through overlapping its genes[86]. The overlapping region of Vpu and Env is one of promising aspect to consider when we focus on Vpu variation. Because host immune responses (neutralizing antibodies) contribute to Env polymorphic nature[87,88], it is enticing to assume that immune responses directed towards Env may influence Vpu polymorphisms through Vpu-Env overlapping region. KIR associated polymorphisms within Vpu-Env overlapping region have been reported previously[44]. Although it is still unknown whether NK cells recognize Vpu or Env protein, nonetheless these findings indicate the importance of this region for Vpu variability. Furthermore, it is reported that X4- and R5-tropic HIV-1 showed differential amino acid polymorphisms in Vpu[89], suggesting that cellular compartment influences Vpu variability.

The current increase in number of new findings of

Vpu from pandemic HIV-1 group M strain and other HIV-1 strains, enlighten us the precise role or mechanisms of how Vpu degrade the viral receptor CD4, antagonize tetherin/BST-2, enhance p53 stability and modulate NK-cell activities through modulation of PVR, NTB-A and CD1d receptors (Figure 3). Understanding the mode of action of Vpu and association of the immune factors certainly open plenty of new windows to deciphering the intricate mechanisms associated with HIV-1 immune pathogenesis *in vivo*. Also, understanding pathways of Vpu intra- and inter-patients sequence variability and adaptation may provide us with an alternative approach for prospects of viral persistence and Vpu contributions *in vivo*.

ACKNOWLEDGMENTS

The authors wish to thank the Ministry of Education, Science, Sports, and Culture (MEXT) of Japan and the Ministry of Health, Labor, and Welfare of Japan for their grant-in aid for the AIDS research. We also wish to thank Dr. J Carlson (Microsoft Research, Redmond, Washington, United States) and M. Mahiti (Kumamoto University, Japan) for their helpful discussion.

REFERENCES

1 **Ji JP**, Loeb LA. Fidelity of HIV-1 reverse transcriptase copying RNA in vitro. *Biochemistry* 1992; **31**: 954-958 [PMID: 1370910]
2 **Shriner D**, Rodrigo AG, Nickle DC, Mullins JI. Pervasive genomic recombination of HIV-1 in vivo. *Genetics* 2004; **167**: 1573-1583 [PMID: 15342499 DOI: 10.1534/genetics.103.023382]
3 **Ramratnam B**, Bonhoeffer S, Binley J, Hurley A, Zhang L, Mittler JE, Markowitz M, Moore JP, Perelson AS, Ho DD. Rapid production and clearance of HIV-1 and hepatitis C virus assessed by large volume plasma apheresis. *Lancet* 1999; **354**: 1782-1785 [PMID: 10577640 DOI: 10.1016/S0140-6736(99)02035-8]
4 **Snoeck J**, Fellay J, Bartha I, Douek DC, Telenti A. Mapping of positive selection sites in the HIV-1 genome in the context of RNA and protein structural constraints. *Retrovirology* 2011; **8**: 87 [PMID: 22044801 DOI: 10.1186/1742-4690-8-87]
5 **O'Brien SJ**, Nelson GW. Human genes that limit AIDS. *Nat Genet* 2004; **36**: 565-574 [DOI: 10.1038/Ng1369]
6 **An P**, Winkler CA. Host genes associated with HIV/AIDS: advances in gene discovery. *Trends Genet* 2010; **26**: 119-131 [PMID: 20149939 DOI: 10.1016/j.tig.2010.01.002]
7 **Brumme ZL**, Walker BD. Tracking the culprit: HIV-1 evolution and immune selection revealed by single-genome amplification. *J Exp Med* 2009; **206**: 1215-1218 [PMID: 19487418]
8 **Brumme ZL**, Art FYP, Jonathan MC, Walkerd BD. Identifying HLA-Associated Polymorphisms in HIV-1. HIV Molecular Immunology 2010. Los Alamos, New Mexico: Los Alamos National Laboratory, Theoretical Biology and Biophysics, 2010: 3-16
9 **Moore CB**, John M, James IR, Christiansen FT, Witt CS, Mallal SA. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* 2002; **296**: 1439-1443 [PMID: 12029127 DOI: 10.1126/science.1069660]
10 **Maldarelli F**, Chen MY, Willey RL, Strebel K. Human immunodeficiency virus type 1 Vpu protein is an oligomeric type I integral membrane protein. *J Virol* 1993; **67**: 5056-5061 [PMID: 8331740]
11 **Schubert U**, Antón LC, Bacík I, Cox JH, Bour S, Bennink JR, Orlowski M, Strebel K, Yewdell JW. CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway. *J Virol* 1998; **72**: 2280-2288 [PMID: 9499087]
12 **Tiganos E**, Yao XJ, Friborg J, Daniel N, Cohen EA. Putative alpha-helical structures in the human immunodeficiency virus type 1 Vpu protein and CD4 are involved in binding and degradation of the CD4 molecule. *J Virol* 1997; **71**: 4452-4460 [PMID: 9151836]
13 **Willey RL**, Maldarelli F, Martin MA, Strebel K. Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes. *J Virol* 1992; **66**: 226-234 [PMID: 1727486]
14 **Dubé M**, Roy BB, Guiot-Guillain P, Mercier J, Binette J, Leung G, Cohen EA. Suppression of Tetherin-restricting activity upon human immunodeficiency virus type 1 particle release correlates with localization of Vpu in the trans-Golgi network. *J Virol* 2009; **83**: 4574-4590 [PMID: 19244337]
15 **Dubé M**, Roy BB, Guiot-Guillain P, Binette J, Mercier J, Chiasson A, Cohen EA. Antagonism of tetherin restriction of HIV-1 release by Vpu involves binding and sequestration of the restriction factor in a perinuclear compartment. *PLoS Pathog* 2010; **6**: e1000856 [PMID: 20386718 DOI: 10.1371/journal.ppat.1000856]
16 **Dube M**, Paquay C, Roy BB, Bego MG, Mercier J, Cohen EA. HIV-1 Vpu Antagonizes BST-2 by Interfering Mainly with the Trafficking of Newly Synthesized BST-2 to the Cell Surface. *Traffic* 2011; **12**: 1714-1729 [DOI: 10.1111/j.1600-0854.2011.01277.x]
17 **Schmidt S**, Fritz JV, Bitzegeio J, Fackler OT, Keppler OT. HIV-1 Vpu blocks recycling and biosynthetic transport of the intrinsic immunity factor CD317/tetherin to overcome the virion release restriction. *MBio* 2011; **2**: e00036-e00011 [PMID: 21610122 DOI: 10.1128/mBio.00036-11]
18 **Sauter D**, Unterweger D, Vogl M, Usmani SM, Heigele A, Kluge SF, Hermkes E, Moll M, Barker E, Peeters M, Learn GH, Bibollet-Ruche F, Fritz JV, Fackler OT, Hahn BH, Kirchhoff F. Human tetherin exerts strong selection pressure on the HIV-1 group N Vpu protein. *PLoS Pathog* 2012; **8**: e1003093 [PMID: 23308067 DOI: 10.1371/journal.ppat.1003093]
19 **Brumme ZL**, John M, Carlson JM, Brumme CJ, Chan D, Brockman MA, Swenson LC, Tao I, Szeto S, Rosato P, Sela J, Kadie CM, Frahm N, Brander C, Haas DW, Riddler SA, Haubrich R, Walker BD, Harrigan PR, Heckerman D, Mallal S. HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins. *PLoS One* 2009; **4**: e6687 [PMID: 19690614 DOI: 10.1371/journal.pone.0006687]
20 **Carlson JM**, Brumme CJ, Martin E, Listgarten J, Brockman MA, Le AQ, Chui CK, Cotton LA, Knapp DJ, Riddler SA, Haubrich R, Nelson G, Pfeifer N, Deziel CE, Heckerman D, Apps R, Carrington M, Mallal S, Harrigan PR, John M, Brumme ZL. Correlates of protective cellular immunity revealed by analysis of population-level immune escape pathways in HIV-1. *J Virol* 2012; **86**: 13202-13216 [PMID: 23055555]
21 **Ueno T**, Motozono C, Dohki S, Mwimanzi P, Rauch S, Fackler OT, Oka S, Takiguchi M. CTL-mediated selective pressure influences dynamic evolution and pathogenic functions of HIV-1 Nef. *J Immunol* 2008; **180**: 1107-1116 [PMID: 18178851]
22 **Wei X**, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM. Antibody neutralization and escape by HIV-1. *Nature* 2003; **422**: 307-312 [PMID: 12646921 DOI: 10.1038/nature01470]
23 **Richman DD**, Wrin T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci USA* 2003; **100**: 4144-4149 [PMID: 12644702 DOI: 10.1073/pnas.0630530100]

24 **Yusim K**, Kesmir C, Gaschen B, Addo MM, Altfeld M, Brunak S, Chigaev A, Detours V, Korber BT. Clustering patterns of cytotoxic T-lymphocyte epitopes in human immunodeficiency virus type 1 (HIV-1) proteins reveal imprints of immune evasion on HIV-1 global variation. *J Virol* 2002; **76**: 8757-8768 [PMID: 12163596]

25 **Rossenkhan R**, Novitsky V, Sebunya TK, Musonda R, Gashe BA, Essex M. Viral diversity and diversification of major non-structural genes vif, vpr, vpu, tat exon 1 and rev exon 1 during primary HIV-1 subtype C infection. *PLoS One* 2012; **7**: e35491 [PMID: 22590503 DOI: 10.1371/journal.pone.0035491]

26 **Pickering S**, Hué S, Kim EY, Reddy S, Wolinsky SM, Neil SJ. Preservation of tetherin and CD4 counter-activities in circulating Vpu alleles despite extensive sequence variation within HIV-1 infected individuals. *PLoS Pathog* 2014; **10**: e1003895 [PMID: 24465210 DOI: 10.1371/journal.ppat.1003895]

27 **Matsuda Z**, Chou MJ, Matsuda M, Huang JH, Chen YM, Redfield R, Mayer K, Essex M, Lee TH. Human Immunodeficiency Virus Type-1 Has an Additional Coding Sequence in the Central Region of the Genome. *P Natl Acad Sci USA* 1988; **85**: 6968-6972 [DOI: 10.1073/pnas.85.18.6968]

28 **Schneider T**, Hildebrandt P, Rönspeck W, Weigelt W, Pauli G. The antibody response to the HIV-1 specific "out" (vpu) protein: identification of an immunodominant epitope and correlation of antibody detectability to clinical stages. *AIDS Res Hum Retroviruses* 1990; **6**: 943-950 [PMID: 1697179]

29 **Reiss P**, Lange JMA, Deronde A, Dewolf F, Dekker J, Danner SA, Debouck C, Goudsmit J. Antibody-Response to Viral Protein-U (Vpu) and Protein-R (Vpr) in Hiv-1-Infected Individuals. *J Acq Immun Def Synd* 1990; **3**: 115-122

30 **Kusk P**, Lindhardt BO, Bugge TH, Holmbäck K, Hulgaard EF. Mapping of a new immunodominant human linear B-cell epitope on the vpu protein of the human immunodeficiency virus type 1. *J Acquir Immune Defic Syndr* 1993; **6**: 334-338 [PMID: 7681110]

31 **Chen YM**, Rey WY, Lan YC, Lai SF, Huang YC, Wu SI, Liu TT, Hsiao KJ. Antibody reactivity to HIV-1 Vpu in HIV-1/AIDS patients on highly active antiretroviral therapy. *J Biomed Sci* 2003; **10**: 266-275 [PMID: 12595763]

32 **Jin X**, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, Kostrikis LG, Zhang L, Perelson AS, Ho DD. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* 1999; **189**: 991-998 [PMID: 10075982]

33 **Schmitz JE**, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallon BJ, Ghrayeb J, Forman MA, Montefiori DC, Rieber EP, Letvin NL, Reimann KA. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 1999; **283**: 857-860 [PMID: 9933172]

34 **Altfeld M**, Rosenberg ES. The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1. *Curr Opin Immunol* 2000; **12**: 375-380 [PMID: 10899028]

35 **Streeck H**, Nixon DF. T cell immunity in acute HIV-1 infection. *J Infect Dis* 2010; **202** Suppl 2: S302-S308 [PMID: 20846037 DOI: 10.1086/655652]

36 **Liu Y**, McNevin JP, Holte S, McElrath MJ, Mullins JI. Dynamics of viral evolution and CTL responses in HIV-1 infection. *PLoS One* 2011; **6**: e15639 [PMID: 21283794 DOI: 10.1371/journal.pone.0015639]

37 **Klein MR**, van Baalen CA, Holwerda AM, Kerkhof Garde SR, Bende RJ, Keet IP, Eeftinck-Schattenkerk JK, Osterhaus AD, Schuitemaker H, Miedema F. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J Exp Med* 1995; **181**: 1365-1372 [PMID: 7699324]

38 **Deeks SG**, Walker BD. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. *Immunity* 2007; **27**: 406-416 [PMID: 17892849]

39 **Miura T**, Brockman MA, Schneidewind A, Lobritz M, Pereyra F, Rathod A, Block BL, Brumme ZL, Brumme CJ, Baker B, Rothchild AC, Li B, Trocha A, Cutrell E, Frahm N, Brander C, Toth I, Arts EJ, Allen TM, Walker BD. HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte [corrected] recognition. *J Virol* 2009; **83**: 2743-2755 [PMID: 19116253]

40 **Messaoudi I**, Guevara Patiño JA, Dyall R, LeMaoult J, Nikolich-Zugich J. Direct link between mhc polymorphism, T cell avidity, and diversity in immune defense. *Science* 2002; **298**: 1797-1800 [PMID: 12459592]

41 **Brumme ZL**, Brumme CJ, Chui C, Mo T, Wynhoven B, Woods CK, Henrick BM, Hogg RS, Montaner JS, Harrigan PR. Effects of human leukocyte antigen class I genetic parameters on clinical outcomes and survival after initiation of highly active antiretroviral therapy. *J Infect Dis* 2007; **195**: 1694-1704 [PMID: 17471440]

42 **Brumme ZL**, Brumme CJ, Heckerman D, Korber BT, Daniels M, Carlson J, Kadie C, Bhattacharya T, Chui C, Szinger J, Mo T, Hogg RS, Montaner JS, Frahm N, Brander C, Walker BD, Harrigan PR. Evidence of differential HLA class I-mediated viral evolution in functional and accessory/regulatory genes of HIV-1. *PLoS Pathog* 2007; **3**: e94 [PMID: 17616974]

43 **Sewell AK**, Price DA, Oxenius A, Kelleher AD, Phillips RE. Cytotoxic T lymphocyte responses to human immunodeficiency virus: control and escape. *Stem Cells* 2000; **18**: 230-244 [PMID: 10924089 DOI: 10.1634/stemcells.18-4-230]

44 **Alter G**, Heckerman D, Schneidewind A, Fadda L, Kadie CM, Carlson JM, Oniangue-Ndza C, Martin M, Li B, Khakoo SI, Carrington M, Allen TM, Altfeld M. HIV-1 adaptation to NK-cell-mediated immune pressure. *Nature* 2011; **476**: 96-100 [PMID: 21814282]

45 **Addo MM**, Altfeld M, Rathod A, Yu M, Yu XG, Goulder PJ, Rosenberg ES, Walker BD. HIV-1 Vpu represents a minor target for cytotoxic T lymphocytes in HIV-1-infection. *AIDS* 2002; **16**: 1071-1073 [PMID: 11953475]

46 **Addo MM**, Yu XG, Rosenberg ES, Walker BD, Altfeld M. Cytotoxic T-lymphocyte (CTL) responses directed against regulatory and accessory proteins in HIV-1 infection. *DNA Cell Biol* 2002; **21**: 671-678 [PMID: 12396610 DOI: 10.1089/104454902760330219]

47 **Kloverpris H**, Karlsson I, Bonde J, Thorn M, Vinner L, Pedersen AE, Hentze JL, Andresen BS, Svane IM, Gerstoft J, Kronborg G, Fomsgaard A. Induction of novel CD8+ T-cell responses during chronic untreated HIV-1 infection by immunization with subdominant cytotoxic T-lymphocyte epitopes. *AIDS* 2009; **23**: 1329-1340 [PMID: 19528789 DOI: 10.1097/QAD.0b013e32832d9b00]

48 **Corbet S**, Nielsen HV, Vinner L, Lauemoller S, Therrien D, Tang S, Kronborg G, Mathiesen L, Chaplin P, Brunak S, Buus S, Fomsgaard A. Optimization and immune recognition of multiple novel conserved HLA-A2, human immunodeficiency virus type 1-specific CTL epitopes. *J Gen Virol* 2003; **84**: 2409-2421 [PMID: 12917462]

49 **Kiepiela P**, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, Moodley E, Reddy S, de Pierres C, Mncube Z, Mkhwanazi N, Bishop K, van der Stok M, Nair K, Khan N, Crawford H, Payne R, Leslie A, Prado J, Prendergast A, Frater J, McCarthy N, Brander C, Learn GH, Nickle D, Rousseau C, Coovadia H, Mullins JI, Heckerman D, Walker BD, Goulder P. CD8( ) T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med* 2007; **13**: 46-53 [DOI: 10.1038/Nm1520]

50 **Hasan Z**, Carlson JM, Gatanaga H, Le AQ, Brumme CJ, Oka S, Brumme ZL, Ueno T. Minor contribution of HLA class I-associated selective pressure to the variability of HIV-1 ac-

cessory protein Vpu. *Biochem Biophys Res Commun* 2012; **421**: 291-295 [PMID: 22503975]
51 **Altfeld M**, Addo MM, Eldridge RL, Yu XG, Thomas S, Khatri A, Strick D, Phillips MN, Cohen GB, Islam SA, Kalams SA, Brander C, Goulder PJR, Rosenberg ES, Walker BD, Collaboration HS. Vpr is preferentially targeted by CTL during HIV-1 infection. *J Immunol* 2001; **167**: 2743-2752
52 **Lucchiari M**, Niedermann G, Leipner C, Meyerhans A, Eichmann K, Maier B. Human immune response to HIV-1-Nef. I. CD45RO- T lymphocytes of non-infected donors contain cytotoxic T lymphocyte precursors at high frequency. *Int Immunol* 1994; **6**: 1739-1749 [PMID: 7865467]
53 **Novitsky V**, Cao H, Rybak N, Gilbert P, McLane MF, Gaolekwe S, Peter T, Thior I, Ndung'u T, Marlink R, Lee TH, Essex M. Magnitude and frequency of cytotoxic T-lymphocyte responses: identification of immunodominant regions of human immunodeficiency virus type 1 subtype C. *J Virol* 2002; **76**: 10155-10168 [PMID: 12239290]
54 **Alter G**, Martin MP, Teigen N, Carr WH, Suscovich TJ, Schneidewind A, Streeck H, Waring M, Meier A, Brander C, Lifson JD, Allen TM, Carrington M, Altfeld M. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med* 2007; **204**: 3027-3036 [PMID: 18025129 DOI: 10.1084/jem.20070695]
55 **Funke J**, Dürr R, Dietrich U, Koch J. Natural killer cells in HIV-1 infection: a double-edged sword. *AIDS Rev* 2011; **13**: 67-76 [PMID: 21587340]
56 **Alter G**, Altfeld M. NK cells in HIV-1 infection: evidence for their role in the control of HIV-1 infection. *J Intern Med* 2009; **265**: 29-42 [DOI: 10.1111/j.1365-2796.2008.02045.x]
57 **Stratov I**, Chung A, Kent SJ. Robust NK cell-mediated human immunodeficiency virus (HIV)-specific antibody-dependent responses in HIV-infected subjects. *J Virol* 2008; **82**: 5450-5459 [PMID: 18353957]
58 **Alsmadi O**, Herz R, Murphy E, Pinter A, Tilley SA. A novel antibody-dependent cellular cytotoxicity epitope in gp120 is identified by two monoclonal antibodies isolated from a long-term survivor of human immunodeficiency virus type 1 infection. *J Virol* 1997; **71**: 925-933 [PMID: 8995609]
59 **Yamada T**, Watanabe N, Nakamura T, Iwamoto A. Antibody-dependent cellular cytotoxicity via humoral immune epitope of Nef protein expressed on cell surface. *J Immunol* 2004; **172**: 2401-2406 [PMID: 14764710]
60 **Nomaguchi M**, Fujita M, Adachi A. Role of HIV-1 Vpu protein for virus spread and pathogenesis. *Microbes Infect* 2008; **10**: 960-967 [PMID: 18672082]
61 **Dubé M**, Bego MG, Paquay C, Cohen ÉA. Modulation of HIV-1-host interaction: role of the Vpu accessory protein. *Retrovirology* 2010; **7**: 114 [PMID: 21176220]
62 **Neil SJ**, Zang T, Bieniasz PD. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 2008; **451**: 425-430 [PMID: 18200009]
63 **Vincent MJ**, Raja NU, Jabbar MA. Human immunodeficiency virus type 1 Vpu protein induces degradation of chimeric envelope glycoproteins bearing the cytoplasmic and anchor domains of CD4: role of the cytoplasmic domain in Vpu-induced degradation in the endoplasmic reticulum. *J Virol* 1993; **67**: 5538-5549 [PMID: 8350411]
64 **Bour S**, Schubert U, Strebel K. The human immunodeficiency virus type 1 Vpu protein specifically binds to the cytoplasmic domain of CD4: implications for the mechanism of degradation. *J Virol* 1995; **69**: 1510-1520 [PMID: 7853484]
65 **Mitchell RS**, Katsura C, Skasko MA, Fitzpatrick K, Lau D, Ruiz A, Stephens EB, Margottin-Goguet F, Benarous R, Guatelli JC. Vpu antagonizes BST-2-mediated restriction of HIV-1 release via beta-TrCP and endo-lysosomal trafficking. *PLoS Pathog* 2009; **5**: e1000450 [PMID: 19478868 DOI: 10.1371/journal.ppat.1000450]
66 **Rong L**, Zhang J, Lu J, Pan Q, Lorgeoux RP, Aloysius C, Guo F, Liu SL, Wainberg MA, Liang C. The transmembrane domain of BST-2 determines its sensitivity to down-modulation by human immunodeficiency virus type 1 Vpu. *J Virol* 2009; **83**: 7536-7546 [PMID: 19474106 DOI: 10.1128/JVI.00620-09]
67 **Mangeat B**, Gers-Huber G, Lehmann M, Zufferey M, Luban J, Piguet V. HIV-1 Vpu neutralizes the antiviral factor Tetherin/BST-2 by binding it and directing its beta-TrCP2-dependent degradation. *PLoS Pathog* 2009; **5**: e1000574 [PMID: 19730691 DOI: 10.1371/journal.ppat.1000574]
68 **Mangeat B**, Gers-Huber G, Lehmann M, Zufferey M, Luban J, Piguet V. HIV-1 Vpu neutralizes the antiviral factor Tetherin/BST-2 by binding it and directing its beta-TrCP2-dependent degradation. *PLoS Pathog* 2009; **5**: e1000574 [PMID: 19730691 DOI: 10.1371/journal.ppat.1000574]
69 **Hauser H**, Lopez LA, Yang SJ, Oldenburg JE, Exline CM, Guatelli JC, Cannon PM. HIV-1 Vpu and HIV-2 Env counteract BST-2/tetherin by sequestration in a perinuclear compartment. *Retrovirology* 2010; **7**: 51 [PMID: 20529266 DOI: 10.1186/1742-4690-7-51]
70 **Shah AH**, Sowrirajan B, Davis ZB, Ward JP, Campbell EM, Planelles V, Barker E. Degranulation of natural killer cells following interaction with HIV-1-infected cells is hindered by downmodulation of NTB-A by Vpu. *Cell Host Microbe* 2010; **8**: 397-409 [PMID: 21075351]
71 **Matusali G**, Potestà M, Santoni A, Cerboni C, Doria M. The human immunodeficiency virus type 1 Nef and Vpu proteins downregulate the natural killer cell-activating ligand PVR. *J Virol* 2012; **86**: 4496-4504 [PMID: 22301152]
72 **Moll M**, Andersson SK, Smed-Sörensen A, Sandberg JK. Inhibition of lipid antigen presentation in dendritic cells by HIV-1 Vpu interference with CD1d recycling from endosomal compartments. *Blood* 2010; **116**: 1876-1884 [PMID: 20530791]
73 **Kelly H**, Mandraju R, Coelho-dos-Reis JG, Tsuji M. Effects of HIV-1-induced CD1c and CD1d modulation and endogenous lipid presentation on CD1c-restricted T-cell activation. *BMC Immunol* 2013; **14**: 4 [PMID: 23347583 DOI: 10.1186/1471-2172-14-4]
74 **Flaig RM**, Stark S, Watzl C. Cutting edge: NTB-A activates NK cells via homophilic interaction. *J Immunol* 2004; **172**: 6524-6527 [PMID: 15153464]
75 **Bolduan S**, Hubel P, Reif T, Lodermeyer V, Höhne K, Fritz JV, Sauter D, Kirchhoff F, Fackler OT, Schindler M, Schubert U. HIV-1 Vpu affects the anterograde transport and the glycosylation pattern of NTB-A. *Virology* 2013; **440**: 190-203 [PMID: 23528733 DOI: 10.1016/j.virol.2013.02.021]
76 **Shibuya A**, Campbell D, Hannum C, Yssel H, Franz-Bacon K, McClanahan T, Kitamura T, Nicholl J, Sutherland GR, Lanier LL, Phillips JH. DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity* 1996; **4**: 573-581 [PMID: 8673704]
77 **Takai Y**, Miyoshi J, Ikeda W, Ogita H. Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. *Nat Rev Mol Cell Biol* 2008; **9**: 603-615 [PMID: 18648374 DOI: 10.1038/nrm2457]
78 **Mattner J**, Debord KL, Ismail N, Goff RD, Cantu C, Zhou D, Saint-Mezard P, Wang V, Gao Y, Yin N, Hoebe K, Schneewind O, Walker D, Beutler B, Teyton L, Savage PB, Bendelac A. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 2005; **434**: 525-529 [PMID: 15791258 DOI: 10.1038/nature03408]
79 **Tupin E**, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol* 2007; **5**: 405-417 [PMID: 17487145 DOI: 10.1038/nrmicro1657]
80 **Kawana K**, Matsumoto J, Miura S, Shen L, Kawana Y, Nagamatsu T, Yasugi T, Fujii T, Yang H, Quayle AJ, Taketani Y, Schust DJ. Expression of CD1d and ligand-induced cytokine production are tissue specific in mucosal epithelia of the human lower reproductive tract. *Infect Immun* 2008; **76**: 3011-3018 [PMID: 18458073 DOI: 10.1128/IAI.01672-07]

81 **Verma S**, Ali A, Arora S, Banerjea AC. Inhibition of β-TrcP-dependent ubiquitination of p53 by HIV-1 Vpu promotes p53-mediated apoptosis in human T cells. *Blood* 2011; **117**: 6600-6607 [PMID: 21521785]

82 **Bushman FD**, Hoffmann C, Ronen K, Malani N, Minkah N, Rose HM, Tebas P, Wang GP. Massively parallel pyrosequencing in HIV research. *AIDS* 2008; **22**: 1411-1415 [PMID: 18614863 DOI: 10.1097/QAD.0b013e3282fc972e]

83 **Bimber BN**, Burwitz BJ, O'Connor S, Detmer A, Gostick E, Lank SM, Price DA, Hughes A, O'Connor D. Ultradeep pyrosequencing detects complex patterns of CD8+ T-lymphocyte escape in simian immunodeficiency virus-infected macaques. *J Virol* 2009; **83**: 8247-8253 [PMID: 19515775]

84 **Henn MR**, Boutwell CL, Charlebois P, Lennon NJ, Power KA, Macalalad AR, Berlin AM, Malboeuf CM, Ryan EM, Gnerre S, Zody MC, Erlich RL, Green LM, Berical A, Wang Y, Casali M, Streeck H, Bloom AK, Dudek T, Tully D, Newman R, Axten KL, Gladden AD, Battis L, Kemper M, Zeng Q, Shea TP, Gujja S, Zedlack C, Gasser O, Brander C, Hess C, Günthard HF, Brumme ZL, Brumme CJ, Bazner S, Rychert J, Tinsley JP, Mayer KH, Rosenberg E, Pereyra F, Levin JZ, Young SK, Jessen H, Altfeld M, Birren BW, Walker BD, Allen TM. Whole genome deep sequencing of HIV-1 reveals the impact of early minor variants upon immune recognition during acute infection. *PLoS Pathog* 2012; **8**: e1002529 [PMID: 22412369 DOI: 10.1371/journal.ppat.1002529]

85 **Henn MR**, Boutwell CL, Charlebois P, Lennon NJ, Power KA, Macalalad AR, Berlin AM, Malboeuf CM, Ryan EM, Gnerre S, Zody MC, Erlich RL, Green LM, Berical A, Wang Y, Casali M, Streeck H, Bloom AK, Dudek T, Tully D, Newman R, Axten KL, Gladden AD, Battis L, Kemper M, Zeng Q, Shea TP, Gujja S, Zedlack C, Gasser O, Brander C, Hess C, Günthard HF, Brumme ZL, Brumme CJ, Bazner S, Rychert J, Tinsley JP, Mayer KH, Rosenberg E, Pereyra F, Levin JZ, Young SK, Jessen H, Altfeld M, Birren BW, Walker BD, Allen TM. Whole genome deep sequencing of HIV-1 reveals the impact of early minor variants upon immune recognition during acute infection. *PLoS Pathog* 2012; **8**: e1002529 [PMID: 22412369 DOI: 10.1371/journal.ppat.1002529]

86 **Chirico N**, Vianelli A, Belshaw R. Why genes overlap in viruses. *Proc Biol Sci* 2010; **277**: 3809-3817 [PMID: 20610432]

87 **Fenyö EM**, Albert J, McKeating J. The role of the humoral immune response in HIV infection. *AIDS* 1996; **10** Suppl A: S97-106 [PMID: 8883616]

88 **McKeating JA**. Biological consequences of human immunodeficiency virus type 1 envelope polymorphism: does variation matter? 1995 Fleming Lecture. *J Gen Virol* 1996; **77** (Pt 12): 2905-2919 [PMID: 9000081]

89 **Dimonte S**, Babakir-Mina M, Aquaro S, Perno CF. Specific VpU codon changes were significantly associated with gp120 V3 tropic signatures in HIV-1 B-subtype. *Virol Sin* 2012; **27**: 360-368 [PMID: 23271577 DOI: 10.1007/s12250-012-3287-0]

90 **Efron B**, Tibshirani R. Statistical data analysis in the computer age. *Science* 1991; **253**: 390-395 [PMID: 17746394]

91 **Schubert U**, Strebel K. Differential activities of the human immunodeficiency virus type 1-encoded Vpu protein are regulated by phosphorylation and occur in different cellular compartments. *J Virol* 1994; **68**: 2260-2271 [PMID: 8139011]

P- Reviewer: Brett TJ, Fackler OT **S- Editor**: Wen LL
L- Editor: A **E- Editor**: Wang CH

World Journal of Immunology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5411/wji.v4.i2.116

World J Immunol 2014 July 27; 4(2): 116-121
ISSN 2219-2824 (online)

REVIEW

Targeting TLR4/MAPKs signaling pathway: A better option for therapeutic inhibition of atherosclerosis

Janeesh Plakkal Ayyappan, Annie Abraham

Janeesh Plakkal Ayyappan, Annie Abraham, Department of Biochemistry, School of Life sciences, University of Kerala, Kariavattom Campus, Thiruvananthapuram 695 581, Kerala, India
Author contributions: All authors contributed to this paper.
Supported by University of Kerala, India for the study
Correspondence to: Dr. Annie Abraham, Director, Professor of Biochemistry, Department of Biochemistry, School of Life sciences, University of Kerala, Kariavattom Campus, Thiruvananthapuram 695 581, Kerala, India. annieab2001@gmail.com
Telephone: +91-471-2308078 Fax: +91-471-2308614
Received: March 28, 2014 Revised: May 10, 2014
Accepted: June 27, 2014
Published online: July 27, 2014

Abstract

Cardiovascular diseases, especially atherosclerosis, found to be the dreadful diseases worldwide. There are diverse pathways associated with the progression of atherosclerosis. One of the important signaling pathways to target atherosclerotic plaque rupture is toll-like receptor 4 (TLR4) Pathway. Several studies are available for illustrating the role of TLR4 in health and diseases. Different types of immune cell are activated in atherosclerosis but primary cells that are activated by the TLR4 signaling are macrophages and endothelial cells. Mechanisms by which macrophages uptake lipids are diverse and it is very important to target signaling pathway responsible for controlling foam cell formation. The process of macrophages transformed foam cell formation is the critical event in progression of atherosclerotic lesion and TLR4 found to have actively participate in the event through mitogen activated protein kinases (MAPKs) activation. The activation of MAPKs signaling pathway leads to the accumulation of cholesterol in the macrophages and also contribute to the dissociation of IκB and the nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 subunit, thereby activating key inflammatory cascade activation by MAPKs/NF-κB signaling pathway to induce toxicity by activating different inflammatory parameters. Hence, the review focussed on exploring the role of TLR4/MAPKs signaling pathway for the therapeutic inhibition of atherosclerosis.

Key words: Atherosclerosis; Toll-like receptor 4; Mitogen activated protein kinase; Foam cells; Inflammatory markers

Core tip: The inhibition of atherosclerosis is one of primary target for the therapeutics of cardiovascular diseases, which is the eminent health problem worldwide. The important function of toll-like receptor 4 (TLR4) in the activation and progression of atherosclerosis is justified here. The TLR4 in turn activates the mitogen activated protein kinases (MAPKs) and nuclear factor kappa-light-chain-enhancer of activated B cells which are responsible for most of the inflammatory events. Hence, therapeutic inhibition of TLR4/MAPKs signaling pathway is one of the best method of inhibiting atherosclerosis.

Plakkal Ayyappan J, Abraham A. Targeting TLR4/MAPKs signaling pathway: A better option for therapeutic inhibition of atherosclerosis. *World J Immunol* 2014; 4(2): 116-121 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i2/116.htm DOI: http://dx.doi.org/10.5411/wji.v4.i2.116

INTRODUCTION

Cardiovascular disease, especially atherosclerosis is a main health problem worldwide and it is a disease characterised by the deposition of lipid in the blood vessels. There are several studies undertaken to know the proximal role of

immune system in atherosclerosis[1]. Macrophages are the primary cells which are present in atherosclerotic lesions and they uptake lipids and get transformed to foam cells. These foam cells are risky and contribute to the development of atherosclerotic plaque rupture.

It was known that inflammatory process and its further cascade by activating immune system may contribute to the development of inflammation related atherosclerosis[2]. Usually the luminal side of the blood vessel walls are prone to atherosclerotic injury[3]. The presence of human histocompatibility leukocyte antigen is widely upregulated as the result of inflammatory processes[4]. There are studies reporting the role of variety of Infectious organisms and HSP60 as trigger of atherosclerosis[5].

It is very important to know the mechanism by which macrophages uptakes lipid and transformed get into foam cells. Targeting of macrophages transformed to foam cells are very important therapeutic strategies[6]. The studies on mechanism by which macrophages accumulate OxLDL and its further activation cascades are very important. It usually activates further cascades by activating components like polyoxygenated cholesteryl ester hydroperoxides and in turn activates toll-like receptor 4 (TLR4)[6].

It was suggested that TLR4 act as a link between inflammation and atherosclerosis[7]. TLR4 found to have an active participation in the progression of atherosclerotic diseases. It can also interferes with the cholesterol metabolic machinery in macrophages[8]. The research in TLR4 shown that, silencing of TLR4 gene seems to have reduced the size of atherosclerotic lesion, lipid content and macrophage polarisation in mice fed a high cholesterol diet for continuous six months[9].

TLR4 found to have act as an important receptor for arterial remodelling[10]. The activation of TLR4 receptor leads to the further activation of MYD88 protein and through protein cascade further activates mitogen activated protein kinases (MAPKs). The activation of MAPKs are essential for the secretion of chemoattract protein to direct monocytes to the atherosclerotic site[11]. The study on inhibition of tyrosine phosphatases like MAPKs found to have demolished the atherosclerotic lesion size in mice[11].

The phosphorylation of MAPKs triggers the activation of several downstream proteins and further activates the nuclear factor translocation (NF-κB) which ultimately leading to the progression and rupture of atherosclerotic plaque [12]. Hence, the review focussed on exploring the role of TLR4/MAPKs signaling pathway in therapeutic inhibition of atherosclerosis.

ATHEROSCLEROSIS AND ITS ACTIVATION BY IMMUNE SYSTEM

The immune system is considered to be the guardian of host and its activation as a result to solve the denudation of endothelium. If the immune system unable to control this activation, then it will result in the chronic immune reaction and can result in the development of atherosclerotic plaque formation[13]. The regions of atherosclerotic lesions are usually crowded with macrophages and T cells which usually plays an adequate role in innate and acquired immune reactions. It was known in atherosclerotic disease condition there is an clonal expansion of differentiated T cells, which are common in all adaptive immune reactions[14].

TOLL-LIKE RECEPTOR-4

Toll-like receptor-4 (TLR4) pattern-recognition receptors are found to have an important role in the immune function. TLRs resides in the family of type I transmembrane receptor which consists of intracellular domain and an extracellular leucine repeat domain[15-17]. It was known that human TLR4 was the first characterised form of mammalian toll[15]. TLR4 is expressed in different types of cells, among them most abundant cell type is macrophages and dendric cells[15]. Usually, it is an membrane receptor which act as a signal transducing agent in different inflammatory insult condition like LPS induced[18-21].

The extensive research in the field of TLRs resulted in knowing mechanism of immune response induced by TLR4, it is by recognition the pathogen associated molecular pattern. The recent studies using mouse knock out genes demonstrated the active role of TLR4 in triggering and development of atherosclerotic plaque[15].

TLR4 IN HEALTH AND DISEASES

Among the toll like receptors, the best characterised form is the TLR4, which has found to have prominent role in the atherosclerosis[22]. The tissue slice from aorta of atherosclerotic plaque area showed an prominent expression of TLR4 by immunohistochemical analysis[23].

The research studies on cardiovascular diseases shown that infection associated with C pneumonia found to have role in the progression of atherosclerotic diseases[24]. It usually triggers the diseases by activating TLR4 receptor to induce the migration and proliferation of smooth muscle cells[25]. The patient with up regulated expression of human TLR4 results in the elevation of IL-12 expression on the downstream activation of TLR4[22].

Lipopolysaccharide are released upon microbial infection and might triggers the plaque cells to promote the production of different cytokines which initiates the progression of plaque and its rupture which results in severe complications[26]. The up regulated expression of hTLR4 in patients results in the enhanced expression of MYD88 protein level[27]. Extensive genetic study on TLR4 gene showed that any polymorphism in TLR4 gene found to have slow down the progress of atherosclerosis. It is due to the mutation on TLR4 (Asp 299 Gly and Thr 399ile) residues. The analysis on TLR4 polymorphism in different patient showed that the patient with acute coronary syndrome showed less polymorphism were as healthy old people showed least polymorphism[28].

ACTIVATION OF IMMUNE CELLS BY TLR4 SIGNALING

Macrophages and the endothelial cells are the main two types of cells which primary respond to the microbial infection. TLR4 expression in macrophages triggers the local differentiation of these cells to antigen presenting one[29,30]. Finally it act as the bridge between innate and adaptive immune response to local antigen such as heat shock proteins and OxLDL[31].

TLR4 AND ITS ROLE IN CHOLESTEROL METABOLISM

TLR4 has active role in cholesterol metabolism in macrophages[8], which elucidates the process by which TLR4 affect the disease pathology. It has been found that deficiency in TLR4 gene was associated with reduction in the atherosclerotic lesion in cholesterol fed mice for six months[9]. The gene polymorphism in TLR4 results in the 25% reduction in plaque of double mutant mice. The levels of plasma cholesterol didn't affect significantly on TLR4 deficiency. Over all the genetic polymorphism in TLR4 results in the reduction in levels of cholesterol, conforming the active role of TLR4 in atherosclerosis.

TLR4 SIGNALING IN ATHEROSCLEROSIS

The innate immune system can be activated by variety of pathogen by TLR4 signaling pathways[16,18]. Lipopolysacharide can specifically activates TLR4 ligand[31], which is the major component of gram negative bacteria. Cholesterol induced toxicity causes tissue injury and which releases cellular fibronectin and HSP60 which triggers the activation of TLR4 receptor and results in the atherosclerotic progression[32,33].

The activation of TLR4 leads to the accumulation of different cells in the atherosclerotic walls like endothelial cells[20,30], macrophages[7,20,30], adventitial fibroblast[20,34] and dendric cells[20,35,36]. TLRs have two important domains like extracellular leucine rich (LRR) domain and intracellular domain (TIR). When the TLR4 receptor stimulates, the TIR domain bind to TIR domain adaptor protein MYD88, then to adaptor protein (AD) to form TIRAP complex which is known as MYD88-MAIL and TIR domain consist of adaptor inducing IFN-β (TRIF), the TRIF-related adaptor molecule (TRAM) resulting in two distinct signaling mechanism. MyD88-dependent and the MyD88-independent/TRIF-dependent pathways[37].

MAPKS ACTIVATION BY TLR4 SIGNALING

TLR4 is widely expressed in atherosclerotic plaques and results in the activation of macrophages and endothelial cells. There comes a link between TLR4/MAPKs/NF-κB pathway in inducing inflammatory stress and ultimately resulting in atherosclerotic plaque rupture[30]. Upon activation TLR4 receptor leads to the activation of IRAK associated protein TRAF6 which induces activation of TAK1 and MKK6 *via* JNK/p38 to activates NF-κB and resulting in the activation of downstream signaling to promote the progression of the disease[38,39].

TLR4/MAPKS SIGNALING PATHWAY AS A THERAPEUTIC TARGET

TLR4 found to have an eminent role in the innate immune system. When it comes in with microbial product TLR4 activates intracellular signaling pathway. The execution of the mechanism is through NF-κB signaling pathway. It is known that TLR4 induced NF-κB activation is an critical component in ancient host defence system, which is phylogenetically conserved in most of insects and mammals[40].

The alterations in the mechanisms regulating the activation of MAPKs and NF-κB are responsible for the most of inflammatory events[12]. In normal cells the NF-κB resides in the cytoplasm and usually associated with Iκ-B, a family of inhibitory proteins, which usually binds to NF-κB and inhibits the nuclear translocation[41]. NF-κB usually regulates the cell survival and inflammatory stress on the active κB binding sites called the promoter gene[12]. Active NF-κB complexes are dimers of combinations of Rel family polypeptides (p50, p52 and p65) that respond to a wide variety of stimuli. The NF-κB subunit determines the biological effect by nuclear translocation and further binding to κB-regulatory elements[42,43].

Research study on MAPKs pathway suggests the active participation of MAPKs in the translocation of NF-κB subunits. Upon inflammatory stress the cells elicts inflammatory responses *via* MAPKs signaling pathway. It regulates various cellular activities like gene expression, mitosis, programmed cell death, *etc.* The phosphorylation of MAPKs act as switch for tuning the activation of target protein on/off[44,45].

Natural products have long been recognized as an important source of therapeutically effective medicines. It is recognized that natural-product structures have great chemical diversity, biochemical specificity and other molecular properties that make them favourable lead structures[46]. There are several plant compounds which can be used to target this pathway. We have recently published our research paper on Robinin a bioflavonoid from *Vigna unguiculata* leaf[47,48] which selectively modulates TLR/NF-κB signaling pathway in oxidized LDL induced human peripheral blood mononuclear cells[49]. Targeting of TLR4/MAPKs signaling pathway (Figure 1) is very essential for the therapeutic inhibition of atherosclerosis. The activation of TLR4 in turn activates cascades of proteins and IKK dependent phosphorylation of IκB. There is also an activation of MAPKs which contribute to the dissociation of IκB and the nuclear translocation of NF-κB p65 subunit (Figure 1) resulting in the activation of key

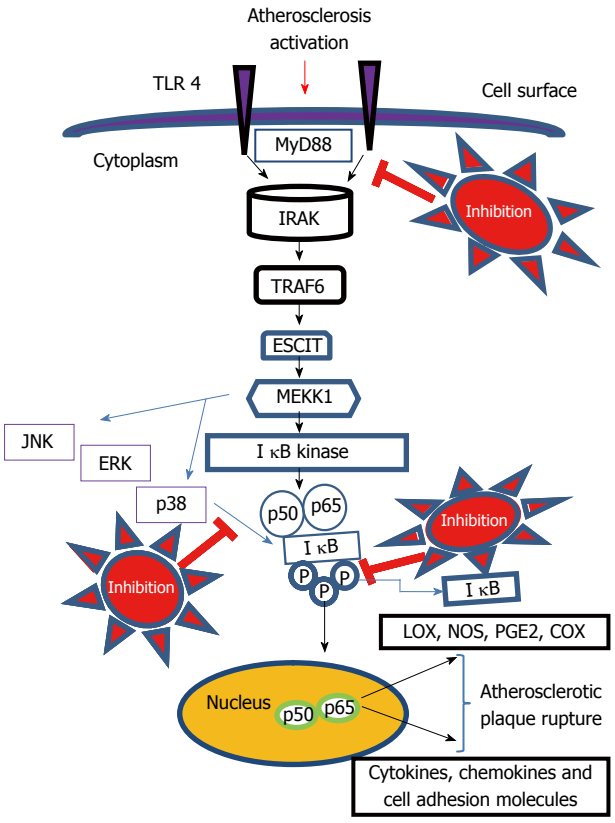


Figure 1 Proposed mechanism of toll-like receptor 4/mitogen activated protein kinases signaling pathway and its suitable targets for therapeutic inhibition of atherosclerosis. The activation of toll-like receptor 4 (TLR4) receptor by various external stimulus leads to the transmittance of signal from cell surface to interior. The TLR4 in turn activates cascades of proteins and finally activates the IKK dependent phosphorylation of IκB and also there is an activation of mitogen activated protein kinase (MAPKs) which also contribute to the dissociation of IκB and the nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 subunit, thereby activating key inflammatory cascade activation by MAPKs/NF-κB signaling pathway to induce toxicity by activating different inflammatory parameters. Hence we can target the TLR4/MAPKs signaling pathway at different places in the signaling pathway which indicated in the diagram. IKK: IκB kinase: JNK- c-Jun N-terminal kinases: ERK: Extracellular signal regulated kinases; MEKK1: Mitogen-activated protein kinase kinase kinase 1.

inflammatory cascade through MAPKs/NF-κB signaling pathway. Hence we can target the TLR4/MAPKs signaling pathway at different places in the signaling pathway as indicated in the proposed mechanism in Figure 1. Hence, Identification of naturally occurring phytocompounds that can suppress or downregulate TLR4/MAPKs signaling pathway would be an efficient strategy for inhibition of atherosclerosis

CONCLUSION

The inhibition of atherosclerosis is one of primary target for the therapeutics of atherosclerosis, the leading cause of death worldwide. The important role of TLR4 in the activation and progression of atherosclerosis is justified here. The TLR4 in turn activates the MAPKs and NF-κB which are responsible for most of inflammatory events. Hence, therapeutic inhibition of TLR4/MAPKs signaling pathway is one of the best method for inhibiting atherosclerosis.

REFERENCES

1 **Hansson GK**, Hermansson A. The immune system in atherosclerosis. *Nat Immunol* 2011; **12**: 204-212 [PMID: 21321594 DOI: 10.1038/ni.2001]
2 **Ross R**. Atherosclerosis--an inflammatory disease. *N Engl J Med* 1999; **340**: 115-126 [PMID: 9887164 DOI: 10.1056/NEJM199901143400207]
3 **Laine P**, Kaartinen M, Penttilä A, Panula P, Paavonen T, Kovanen PT. Association between myocardial infarction and the mast cells in the adventitia of the infarct-related coronary artery. *Circulation* 1999; **99**: 361-369 [PMID: 9918522 DOI: 10.1161/01.CIR.99.3.361]
4 **Ramshaw AL**, Parums DV. Immunohistochemical characterization of inflammatory cells associated with advanced atherosclerosis. *Histopathology* 1990; **17**: 543-552 [PMID: 2076887 DOI: 10.1111/j.1365-2559.1990.tb00794.x]
5 **Xu Q**, Schett G, Li C, Hu Y, Wick G. Mechanical stress-induced heat shock protein 70 expression in vascular smooth muscle cells is regulated by Rac and Ras small G proteins but not mitogen-activated protein kinases. *Circ Res* 2000; **86**: 1122-1128 [PMID: 10850962 DOI: 10.1161/01.CIR.102.1.14]
6 **Miller YI**, Choi SH, Fang L, Harkewicz R. Toll-like receptor-4 and lipoprotein accumulation in macrophages. *Trends Cardiovasc Med* 2009; **19**: 227-232 [PMID: 20382346 DOI: 10.1016/j.tcm.2010.02.001]
7 **Xu XH**, Shah PK, Faure E, Equils O, Thomas L, Fishbein MC, Luthringer D, Xu XP, Rajavashisth TB, Yano J, Kaul S, Arditi M. Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation* 2001; **104**: 3103-3108 [PMID: 11748108 DOI: 10.1161/hc5001.100631]
8 **Castrillo A**, Joseph SB, Vaidya SA, Haberland M, Fogelman AM, Cheng G, Tontonoz P. Crosstalk between LXR and toll-like receptor signaling mediates bacterial and viral antagonism of cholesterol metabolism. *Mol Cell* 2003; **12**: 805-816 [PMID: 14580333 DOI: 10.1016/S1097-2765(03)00384-8]
9 **Michelsen KS**, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, Akira S, Rajavashisth TB, Arditi M. Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. *Proc Natl Acad Sci USA* 2004; **101**: 10679-10684 [PMID: 15249654 DOI: 10.1073/pnas.0403249101]
10 **Hollestelle SC**, De Vries MR, Van Keulen JK, Schoneveld AH, Vink A, Strijder CF, Van Middelaar BJ, Pasterkamp G, Quax PH, De Kleijn DP. Toll-like receptor 4 is involved in outward arterial remodeling. *Circulation* 2004; **109**: 393-398 [PMID: 14699006 DOI: 10.1161/01.CIR.0000109140.51366.72]
11 **Imaizumi S**, Grijalva V, Priceman S, Wu L, Su F, Farias-Eisner R, Hama S, Navab M, Fogelman AM, Reddy ST. Mitogen-activated protein kinase phosphatase-1 deficiency decreases atherosclerosis in apolipoprotein E null mice by reducing monocyte chemoattractant protein-1 levels. *Mol Genet Metab* 2010; **101**: 66-75 [PMID: 20619710]
12 **Garcia-Garcia FJ**, Mullol J, Perez-Gonzalez M, Pujols L, Alobid I, Roca-Ferrer J, Picado C. Signal transduction pathways (MAPKs, NF-κB, and C/EBP) regulating COX-2 expression in nasal fibroblasts from asthma patients with aspirin intolerance. *PLoS One* 2012; **7**: e51281 [PMID: 23240010 DOI: 10.1371/journal.pone.0051281]
13 **Barbic J**, Leef MF, Burns DL, Shahin RD. Role of gamma interferon in natural clearance of Bordetella pertussis infection. *Infect Immun* 1997; **65**: 4904-4908 [PMID: 9393774]
14 **Hansson GK**, Libby P, Schönbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis.

Circ Res 2002; **91**: 281-291 [PMID: 12193460 DOI: 10.1161/01.RES.0000029784.15893.10]
15 **Medzhitov R**, Preston-Hurlburt P, Janeway CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997; **388**: 394-397 [PMID: 9237759]
16 **Hashimoto C**, Hudson KL, Anderson KV. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* 1988; **52**: 269-279 [PMID: 2449285 DOI: 10.1016/0092-8674(88)90516-8]
17 **Rock FL**, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci USA* 1998; **95**: 588-593 [PMID: 9435236]
18 **Poltorak A**, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998; **282**: 2085-2088 [PMID: 9851930 DOI: 10.1126/science.282]
19 **Qureshi ST**, Larivière L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4) *J Exp Med* 1999; **189**: 615-625 [PMID: 9989976 DOI: 10.1084/jem.189.4.615]
20 **Pasterkamp G**, Van Keulen JK, De Kleijn DP. Role of Toll-like receptor 4 in the initiation and progression of atherosclerotic disease. *Eur J Clin Invest* 2004; **34**: 328-334 [PMID: 15147329 DOI: 10.1111/j.1365-2362.2004.01338.x]
21 **Hoshino K**, Tsutsui H, Kawai T, Takeda K, Nakanishi K, Takeda Y, Akira S. Cutting edge: generation of IL-18 receptor-deficient mice: evidence for IL-1 receptor-related protein as an essential IL-18 binding receptor. *J Immunol* 1999; **162**: 5041-5044 [PMID: 10227969]
22 **Andrea D**, Tamara A, Edo D, Snjezana D, Jerko B. 5 Toll-like receptors and atherosclerosis. *Med Glas* 2009; **6**: 23–31
23 **Yamamoto M**, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, Hoshino K, Takeuchi O, Kobayashi M, Fujita T, Takeda K, Akira S. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* 2002; **420**: 324-329 [PMID: 12447441 DOI: 10.1038/nature01182]
24 **Saikku P**. Epidemiologic association of Chlamydia pneumoniae and atherosclerosis: the initial serologic observation and more. *J Infect Dis* 2000; **181** Suppl 3: S411-S413 [PMID: 10839725 DOI: 10.1086/315625]
25 **Sasu S**, LaVerda D, Qureshi N, Golenbock DT, Beasley D. Chlamydia pneumoniae and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ Res* 2001; **89**: 244-250 [PMID: 11485974 DOI: 10.1161/hh1501.094184]
26 **Libby P**, Egan D, Skarlatos S. Roles of infectious agents in atherosclerosis and restenosis: an assessment of the evidence and need for future research. *Circulation* 1997; **96**: 4095-4103 [PMID: 9403635 DOI: 10.1161/01.CIR.96.11.4095]
27 **Björkbacka H**, Kunjathoor VV, Moore KJ, Koehn S, Ordija CM, Lee MA, Means T, Halmen K, Luster AD, Golenbock DT, Freeman MW. Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. *Nat Med* 2004; **10**: 416-421 [PMID: 15034566 DOI: 10.1038/nm1008]
28 **Balistreri CR**, Candore G, Colonna-Romano G, Lio D, Caruso M, Hoffmann E, Franceschi C, Caruso C. Role of Toll-like receptor 4 in acute myocardial infarction and longevity. *JAMA* 2004; **292**: 2339-2340 [PMID: 15547160 DOI: 10.1001/jama.292.19.2339]
29 **Hertz CJ**, Kiertscher SM, Godowski PJ, Bouis DA, Norgard MV, Roth MD, Modlin RL. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *J Immunol* 2001; **166**: 2444-2450 [PMID: 11160304]
30 **Edfeldt K**, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation* 2002; **105**: 1158-1161 [PMID: 11889007]
31 **Kobe B**, Deisenhofer J. Proteins with leucine-rich repeats. *Curr Opin Struct Biol* 1995; **5**: 409-416 [PMID: 7583641]
32 **Aravind L**, Dixit VM, Koonin EV. Apoptotic molecular machinery: vastly increased complexity in vertebrates revealed by genome comparisons. *Science* 2001; **291**: 1279-1284 [PMID: 11181990 DOI: 10.1126/science.291.5507.1279]
33 **Muzio M**, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* 1997; **278**: 1612-1615 [PMID: 9374458]
34 **Vink A**, Schoneveld AH, van der Meer JJ, van Middelaar BJ, Sluijter JP, Smeets MB, Quax PH, Lim SK, Borst C, Pasterkamp G, de Kleijn DP. In vivo evidence for a role of toll-like receptor 4 in the development of intimal lesions. *Circulation* 2002; **106**: 1985-1990 [PMID: 12370224 DOI: 10.1161/01.CIR.0000032146]
35 **Bobryshev YV**, Ikezawa T, Watanabe T. Formation of Birbeck granule-like structures in vascular dendritic cells in human atherosclerotic aorta. Lag-antibody to epidermal Langerhans cells recognizes cells in the aortic wall. *Atherosclerosis* 1997; **133**: 193-202 [PMID: 9298679]
36 **Hoshino K**, Kaisho T, Iwabe T, Takeuchi O, Akira S. Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. *Int Immunol* 2002; **14**: 1225-1231 [PMID: 12356687 DOI: 10.1093/intimm/dxf089]
37 **Falck-Hansen M**, Kassiteridi C, Monaco C. Toll-like receptors in atherosclerosis. *Int J Mol Sci* 2013; **14**: 14008-14023 [PMID: 23880853 DOI: 10.3390/ijms140714008]
38 **Cao Z**, Henzel WJ, Gao X. IRAK: a kinase associated with the interleukin-1 receptor. *Science* 1996; **271**: 1128-1131 [PMID: 8599092 DOI: 10.1126/science.271.5252.1128]
39 **Wang C**, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* 2001; **412**: 346-351 [PMID: 11460167 DOI: 10.1038/35085597]
40 **Zhang G**, Ghosh S. Toll-like receptor-mediated NF-kappaB activation: a phylogenetically conserved paradigm in innate immunity. *J Clin Invest* 2001; **107**: 13-19 [PMID: 11134172]
41 **Baldwin AS**. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 1996; **14**: 649-683 [PMID: 8717528 DOI: 10.1146/annurev.immunol.14.1.649]
42 **Chun KS**, Surh YJ. Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. *Biochem Pharmacol* 2004; **68**: 1089-1100 [PMID: 15313405]
43 **Syeda F**, Grosjean J, Houliston RA, Keogh RJ, Carter TD, Paleolog E, Wheeler-Jones CP. Cyclooxygenase-2 induction and prostacyclin release by protease-activated receptors in endothelial cells require cooperation between mitogen-activated protein kinase and NF-kappaB pathways. *J Biol Chem* 2006; **281**: 11792-11804 [PMID: 16467309 DOI: 10.1074/jbc.M509292200]
44 **Dong C**, Davis RJ, Flavell RA. MAP kinases in the immune response. *Annu Rev Immunol* 2002; **20**: 55-72 [PMID: 11861597 DOI: 10.1146/annurev.immunol.20.091301.131133]
45 **Raman M**, Chen W, Cobb MH. Differential regulation and properties of MAPKs. *Oncogene* 2007; **26**: 3100-3112 [PMID: 17496909 DOI: 10.1038/sj.onc.1210392]
46 **Souto AL**, Tavares JF, da Silva MS, Diniz Mde F, de Athayde-Filho PF, Barbosa Filho JM. Anti-inflammatory activity of alkaloids: an update from 2000 to 2010. *Molecules* 2011; **16**: 8515-8534 [PMID: 21989312 DOI: 10.3390/molecules16108515]
47 **Janeesh PA**, Abraham A. Amelioration of cholesterol induced atherosclerosis by normalizing gene expression, cholesterol profile and antioxidant enzymes by Vigna unguiculata. *Plant Foods Hum Nutr* 2013; **68**: 118-123 [PMID: 23475595

DOI: 10.1007/s11130-013-0345-1]

48 **Janeesh PA**, Abraham A. Vigna unguiculata modulates cholesterol induced cardiac markers, genotoxicity and gene expressions profile in an experimental rabbit model. *Food Funct* 2013; **4**: 568-574 [PMID: 23641512 DOI: 10.1039/c3fo30194j]

49 **Janeesh PA**, Sasikala V, Dhanya CR, Abraham A. Robinin modulates TLR/NF-κB signaling pathway in oxidized LDL induced human peripheral blood mononuclear cells. *Int Immunopharmacol* 2014; **18**: 191-197 [PMID: 24295649 DOI: 10.1016/j.intimp.2013.11.023]

P- Reviewer: Amiya E, Lin GM, Shi GY **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Wang CH

World Journal of Immunology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5411/wji.v4.i2.122

World J Immunol 2014 July 27; 4(2): 122-129
ISSN 2219-2824 (online)

Biologic response modifiers in retinal vasculitis

Sandeep Saxena, Khushboo Srivastav

Sandeep Saxena, Khushboo Srivastav, Retina Service, Department of Ophthalmology, King George's Medical University, Lucknow 226003, India
Author contributions: Saxena S and Srivastav K solely contributed to this paper.
Correspondence to: Sandeep Saxena, MS, FRCS, Retina Service, Department of Ophthalmology, King George's Medical University, Chowk, Lucknow 226003, India. sandeepsaxena2020@yahoo.com
Telephone: +91-94-15160528 Fax: +91-94-15160528
Received: March 29, 2014 Revised: May 11, 2014
Accepted: June 18, 2014
Published online: July 27, 2014

Abstract

Intraocular inflammation is an important cause of blindness both in the developing and developed world. Corticosteroids play a pivotal role in the treatment of intraocular inflammation. Lately, therapy by immunosuppression has taken the center stage for patients with severe intraocular inflammation. However, the side effects of immunosuppressive drugs are oncogenic, infectious, and hematological. Recently, biologic response modifiers specifically targeting suppression of the immune effector responses have revolutionized the treatment of intraocular inflammation. Anti-tumour necrosis factor agents are etanercept, infliximab, and adalimumab. Newer drugs include certolizumab and golimumab. Infliximab has been found to be superior to corticosteroids in treating retinal vasculitis. Anti-interlenkin therapies include rituximab, daclizumab, anakinra, tocilizumab and secukinumab. Rituximab has been proven to be quite effective. Other biologics used are interferons and abatacept. However, there are several limitations and side effects associated with their use.

Key words: Uveitis; Immunotherapy; Biologic response modifiers; Retinal vasculitis; Non-infectious uveitis

Core tip: Corticosteroids play a pivotal role in the treatment of intraocular inflammation. Lately, therapy by immunosuppression has taken the center stage for patients with severe intraocular inflammation. However, biologic response modifiers specifically targeting suppression of the immune effector responses have revolutionized the treatment of intraocular inflammation.

Saxena S, Srivastav K. Biologic response modifiers in retinal vasculitis. *World J Immunol* 2014; 4(2): 122-129 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i2/122.htm DOI: http://dx.doi.org/10.5411/wji.v4.i2.122

INTRODUCTION

Intraocular inflammation accounts for 5% to 20% of blindness in the developed world and 25% in the developing world[1]. Though the prevalence of retinal vasculitis is less, still the complexity and heterogeneity of the disease makes it unique. The etiology of most of them is unknown. Uveitogenic proteins that can incite intraocular inflammation include rhodopsin, retinal arrestin, recoverin, phosducin, retinal pigment epithelium derived (RPE-65) and inter-photoreceptor retinoid binding protein. These uveitogenic retinal antigens incite innate immunity by antigen mimicry and have been found to be associated in patients with intraocular inflammatory disease by numerous studies. Involved immunogenic pathway is similar for all types of intraocular inflammation[2,3]. Over the last two decades, laboratory diagnostic tools have entered into an era of molecular diagnostic tests. With the advent of experimental and cellular biology, several biomarkers are being identified. Many uveitic diseases are known to be strongly associated with particular human leucocyte antigen (HLA) haplotypes. It has largely been supported by continued development of experimental models of autoimmune uveitis along with improved molecular biologic techniques. Novel sophisticated technolo-

gies such as multiplex bead assays have revolutionized the management of complex refractory uveitis. Despite the varied immune etiology, intraocular inflammation poses a significant therapeutic challenge given the heterogeneity of the retinal vasculitis spectrum along with the pressing need and increasing expectations for personalised care. This review attempts to present the current concepts of immunotherapy in retinal vasculitis.

PHYSIOLOGICAL AND PATHOLOGICAL MECHANISM

Immune privilege guards the eye by mechanical sequestration behind an efficient blood-retinal barrier, local inhibition of activation and functioning of adaptive and innate immune cells, and systemic regulation by induction of T regulatory cells[2]. On the other hand, it leaves the eye vulnerable to an autoimmune attack by lymphocytes primed elsewhere in the body by chance encounter with a self or with mimic antigens.

Immunohistologically, retinal vasculitis is characterized by an infiltration of mainly cluster differentiation 4 ($CD4^+$) T cells. Posterior uveitis in humans is considered to be a T cell-mediated autoimmune disease. Importance of T cells is highlighted by the fact that cyclosporin A can be effective in arresting the disease progression in many cases[4]. In an experimental model, the ability to adaptively transfer disease using activated retinal antigen-specific $CD4^+$ T cells is further evidence of $CD4^+$ T cell-mediated processes inducing the irreversible destruction of the photoreceptor cells of the retina[5]. The CD4 interacts directly with major histocompatibility complex (MHC) class Ⅱ molecules on the surface of the antigen-presenting cell. Recognition of the MHC peptide complex by $CD4^+$ T cells leads to secretion of cytokines. T Helper cells (Th) were divided into two subsets: Th1 and Th2. Th1 subset secretes Interferon-γ (IFN-γ) and Interleukin-2 (IL-2) responsible for cellular anti-viral immunity, and a Th2 subset secretes IL-4 required for blood borne parasitic responses. $CD4^+$ Th1 cells and IFN-γ are considered to be the major effectors in the pathogenesis of experimental autoimmune uveitis[6]. Another subset of regulatory $CD4^+$ T cells that secrete IL-10 and transforming growth factor-β (TGF-β) was added[7]. However, the presence of inflammatory diseases in IFN-γ-deficient mice indicated existence of other Th cell subsets and led to the discovery of the Th17 subset secreting IL-17 and IL-23[8]. Recently, other Th cell subsets have been assigned on the basis of the secretion of IL-9 (Th9) or IL-21 (T follicular helper)[9].

CYTOKINE PROFILE IN RETINAL VASCULITIS

Ooi *et al*[10] conducted a systematic review on inflammatory cytokines in uveitis of various etiologies. Few studies were conducted by us to ascertain the cytokine profile in Eales'disease. Following is a description of the cytokines involved in some of the important causes of retinal vasculitis.

Eales' disease (retinal periphlebitis)

Eales' disease is an idiopathic obliterative vasculopathy that primarily affects the peripheral retina of young adults. Role of tumor necrosis factor-alpha (TNF-α) in Eales' disease was evaluated by us in several studies. In one such study, quantification of the TNF-α levels was carried out in young adults with Eales' disease and healthy controls of similar age. TNF-α level was found to be significantly raised in cases as compared with controls. It was also observed that higher levels of TNF-α were associated with increased severity of Eales' disease which was graded according to a new grading system based on severity of inflammation[11]. In another study, we evaluated the levels of TNF in the serum of 52 patients with proliferative stage of Eales' disease and in 32 healthy controls to study its relation with the area of retinal capillary non-perfusion (ischemic retina). TNF levels were significantly increased in the proliferative stage of the disease as compared to controls and higher levels were associated with an increased area of retinal capillary non-perfusion on fluorescein angiography. It was concluded that increased TNF level in proliferative Eales' disease is related to retinal cell death signaling[12]. We conducted another study in which we for the first time evaluated IL-1β, IL-6, IL-10, and TNF-α in the serum of 45 consecutive patients with Eales' disease and in 28 healthy controls. It was found that levels of IL-1β, IL-6, IL-10, and TNF-α were significantly increased in the inflammatory stage of Eales' disease as compared to controls. Also it was observed that IL-1β levels decreased significantly and TNF-α levels increased significantly during the proliferative stage of the disease as compared to the inflammatory stage. It was concluded that for controlling inflammatory activity and/or the associated long-term sequelae related to angiogenesis in Eales' disease, IL-1 system and TNF-α represent novel target for immunotherapy[13].

Behcet's disease

It is a systemic vasculitis with recurrent ocular involvement as uveitis and retinal vasculitis. HLA-B51 phenotype association has been found. Raised intraocular levels of the following immune factors have been found: IL-2, IL-6, IFN-γ and TNF-α. Recurrent episodes of Behcet' s disease-related uveitis has been found to be positively correlated with serum TNF-α levels[14].

Sarcoidosis

An acute or chronic granulomatous uveitis of unknown etiology involving the anterior, intermediate or posterior uveal layers. The aqueous immune profile of patients with sarcoidosis revealed elevated levels of IL-1α, IL-6 and IL-8[10,15].

Vogt-koyanagi-harada disease

A multisystem chronic granulomatous disorder associated

with HLA-DR1 and HLA-DR4 phenotype with ocular manifestation as a chronic, bilateral panuveitis. Raised intraocular levels of the following immune factors have been identified: IL-6, IL-8 and IFN-γ[16].

Fuchs' heterochromic iridocyclitis

A chronic typically unilateral anterior uveitis syndrome with or without associated glaucoma. One study found IFN-γ to be raised in aqueous samples of patients with FHC when compared to patients with idiopathic uveitis. Higher levels of IL-10 was found in larger number of FHC samples than of idiopathic uveitis (not statistically significant)[17].

Idiopathic uveitis

The commonest form of uveitis and has been found to be associated with increased intraocular levels of IL-1β, IL-2, TNF-α, IFN-γ, IL-6, IL-8 and MCP-1[16,18].

Ankylosing spondylitis

A chronic inflammatory disorder of the axial skeleton with a strong association with HLA-B27 phenotype which manifests in the eye as severe acute anterior uveitis. Reports have revealed elevated intraocular levels of IL-2, IFN-γ, IL-6 and TNF-α[15].

IMMUNOTHERAPY

Corticosteroids and immunosuppressants

Corticosteroids played a pivotal role in the treatment of intraocular inflammation in the early 1950s, later on therapy by immunosuppression took the center stage for patients with severe intraocular inflammation. Now with the proteomic labeling, we can target specific cytokine pathway and deliver targeted therapy for patients with intraocular inflammation. We have now probably embarked on much specialised stratified care[4,5,18-23]. The treatment of noninfectious posterior uveitis can lead to severe vision loss, and the first-line conventional treatment includes systemic steroids. When the prednisone doses necessary to control intraocular inflammation are above 0.3 mg/d, a therapeutic association is proposed in order to lower the daily prednisone dose. The combined drugs are immunosuppressive or immunomodulative. The side effects of immunosuppressive drugs are oncogenic, infectious, hematological and can involve reproductive troubles, associated with specific toxic effects depending on the drug used. We undertook a tertiary care center-based prospective interventional study to evaluate the response time and safety profile of low-dose oral methotrexate pulsed therapy in Eales' disease. Twenty one consecutive patients with idiopathic retinal periphlebitis were administered 12.5 mg methotrexate as a single oral dose, once per week for 12 wk. Drug safety was monitored by various laboratory tests that included twice-weekly white blood cells and differential counts, twice-weekly platelet counts, and monthly liver function tests for a mean follow-up period of 6 mo. It was found that all patients showed improvement in visual acuity. All the side effects of methotrexate were mild to moderate in severity and rapidly reversible on dose reduction or discontinuation. We concluded that low dose oral methotrexate pulse therapy (at a dose of 12.5 mg/wk) is clinically effective within 4 wk and is associated with an acceptable safety profile[24]. Conventional therapy with corticosteroids and immunosuppressive agents (such as methotrexate, azathioprine, mycophenolate mofetil and cyclosporine) may not be sufficient to control ocular inflammation or prevent non-ophthalmic complications in refractory patients. In a study conducted by us, efficacy of combined oral corticosteroid and low-dose oral methotrexate pulsed therapy in Eales' disease was evaluated prospectively based on weighted visual morbidity scale for disease activity and visual acuity grading in 36 consecutive cases. Oral corticosteroids in a weekly tapering dose for 4 wk and 12.5 mg methotrexate as a single oral dose, once per week for 12 wk were administered simultaneously. We concluded that this combined oral therapy is clinically effective with an acceptable safety profile[25].

Biologic response modifiers

Biologics specifically target inflammatory cytokines and cause suppression of the immune effector responses that are responsible for damaging tissues. They were first used for ocular inflammation in 1990s. Commonly used biologics are anti-TNF agents and anti-interleukins. Now we have entered into an era of recombinant cytokines.

Anti TNF-α agents

TNF-α is a pleiotropic inflammatory cytokine. It plays a pivotal role in down-regulating both inflammatory and the immune response. Thus, blockade with anti-TNF agents has turned into the most important tool in the management of retinal vasculitis. The three most commonly used TNF inhibitors in the US are infliximab, etanercept and adalimumab. Newer drugs include certolizumab and golimumab.

Infliximab: Infliximab is a chimeric immunoglobulin G1 (IgG1) monoclonal antibody with the antigen-binding region derived from a mouse antibody and the constant region from a human antibody[26]. It binds to TNF-α with high affinity thereby blocking the binding of TNF-α to its receptor. One of the considerations in giving infliximab is that it can potentially induce antinuclear antibody and anti-double stranded DNA on long term therapy[27,28]. Early monitoring and optimizing dose regimens can be useful in patients on long term infliximab therapy. Side effects are autoimmune diseases which improve on stopping the drug, blood dyscrasias, allergies secondary to infusion, fever, fatigue, upper respiratory chest infection, headache, gastrointestinal upset, headache.

Adalimumab: Adalimumab is a fully humanized recombinant IgG1 monoclonal antibody with high binding to

human TNF-α. Side effects are gastrointestinal disturbances including haemorrhage, hyperlipidaemia, hypertension, chest pain, tachycardia, cough, dyspnea, mood changes, paraesthesia, haematuria, renal impairment, electrolyte disturbances, hyperuricaemia, musculoskeletal pain, eye disorders (visual impairment, conjunctivitis, blepharitis, eye swelling), rash, dermatitis.

Etanercept: Etanercept is a soluble fusion protein and prevents both TNF-α and TNF-β from interacting with receptors. It consists of 2 dimers of higher affinity type 2 TNF receptors. Side effects include headache, infection like upper respiratory tract infections, urinary tract infections, butterfly rash on cheeks, dizziness, fatigue, swelling of the arms/legs, unusual bruising/bleeding, severe headache, mental/mood changes, seizures, unexplained muscle weakness, numbness/tingling of the hands/feet, unsteadiness, vision changes, severe stomach/abdominal pain.

Golimumab: Golimumab is a novel fully humanized anti-TNFα monoclonal antibody. Side effects include body aches or pain, chills, cough, difficulty with breathing, ear congestion, fever, headache, loss of voice, muscle aches, sneezing, sore throat, stuffy or runny nose, unusual tiredness or weakness. Blurred vision, burning, crawling, itching, numbness, prickling, "pins and needles", or tingling feelings, congestion cough with mucus diarrhea, dizziness, general feeling of discomfort or illness, hoarseness, joint pain, loss of appetite, muscle aches and pains, nausea, nervousness, pain or tenderness around the eyes and cheek bones, painful cold sores or blisters on the lips, pounding in the ears, shivering, shortness of breath or troubled breathing, slow or fast heartbeat, sweating, tender/swollen glands in the neck.

Anti-TNF-α agents have improved the treatment armamentarium for refractory immune-mediated uveitis particularly in Behçet disease-associated uveitis. A prospective observational study of patients with panuveitis was undertaken in which 19 eyes received an infliximab infusion, 8 eyes received high-dose methylprednisolone intravenously and 8 eyes received intravitreal triamcinolone acetonide at attack's onset. Unchanged baseline maintenance therapy was continued for 30 d. Visual acuity, anterior chamber cells, vitreous cells and inflammation of the posterior eye segment were assessed at baseline and at days 1, 7, 14 and 29 post-treatment. Infliximab was superior to corticosteroids in treating retinal vasculitis as well as in resolution of retinitis and cystoid macular oedema[29]. A study was conducted in which anti-TNFα therapy was administered in 15 patients of chronic non-infectious uveitis when no response had been obtained with classical immunosuppressive therapies or in the presence of severe rheumatoid disease. Mean duration of ocular disease was 8 years. Treatment was initiated with infliximab, etanercept, and adalimumab. It was concluded that anti-TNF-α therapy is effective and safe[30]. Importance of TNF-α in the pathophysiology of multi-systemic sarcoidosis and refractory retinal vasculitis was emphasized in a case report in which 2 patients experienced an excellent response to infliximab[31]. A retrospective noncomparative case series was conducted on 6 pediatric patients with uveitis refractory to methotrexate, cyclosporine, mycophenolate mofetil, etanercept, daclizumab and topical steroids. These patients initially received infliximab at doses between 5 and 10 mg/kg at 2 to 4 wk interval and then were maintained at 4 to 8 wk interval at doses of 5 to 18 mg/kg. Reduction in intraocular inflammation after infliximab therapy initiation was seen in all the patients. The only adverse reactions seen were vitreous hemorrhage in 1 patient and a case of transient upper respiratory infusion reaction. It was concluded that for the treatment of refractory pediatric uveitis, infliximab seems to be an effective agent without apparent serious toxicity[32]. To evaluate the clinical response after switching from infliximab to adalimumab, a prospective, longitudinal and observational study was conducted in 69 patients with Behcet's disease. Seventeen patients were switched to adalimumab for lack or loss of efficacy or infusion reactions to infliximab. Of the 17 treated patients, 9 showed sustained remission of the disease and 3 showed good response. No side effects were observed in any patient. They concluded that adalimumab can be used to treat patients with Behcet's disease showing a scarce response or adverse events to infliximab[33]. A study was conducted to alert physician for timely recognition and to evaluate current treatment of recurrent hypopyon iridocyclitis or panuveitis in Behçet's disease. It was found that for the control of acute panuveitis, a single infliximab infusion should be considered, whereas in reducing the number of episodes in refractory uveoretinitis with faster regression and for complete remission of cystoid macular edema, repeated long-term infliximab infusions proved to be more effective[34]. Rifkin *et al*[35] studied current status of three of the five commercially available TNF inhibitors-etanercept, infliximab, and adalimumab for their efficacy in treatment of ocular inflammation. They found etanercept to be inadequate in controlling ocular inflammation. Infliximab and adalimumab, however, showed encouraging results in multiple trials[35]. There are only two reports in the literature about the use of golimumab in uveitis, describing four patients with juvenile idiopathic arthritis-associated uveitis and a case of idiopathic retinal vasculitis. Mesquida *et al*[36] first reported about the use of golimumab in Behçet's disease. William *et al*[37] reported good outcomes using golimumab in three patients with juvenile idiopathic arthritis.

Anti-interleukin therapies

Rituximab: Rituximab (first used in the treatment of Non Hodgkin's B cell lymphoma) is a recombinant chimeric monoclonal antibody with binding efficacy to CD20. It works by blocking CD20-bearing B cells. Side effects are severe stomach pain with constipation, bloody or tarry stools, coughing up blood or vomit that looks like coffee grounds, painful blistering skin rash with

burning, itching, or tingly feeling, or upper stomach pain, vomiting, loss of appetite, dark urine, clay-colored stools, jaundice (yellowing of the skin or eyes), runny or stuffy nose, sinus pain, sore throat, headache, dizziness, itching, or mild stomach cramps.

Daclizumab: Daclizumab is a recombinant monoclonal antibody of the human IgG1 isotype composed of 90% human and 10% mouse antibody sequences that bind to CD25 with high affinity and inhibit IL-2-mediated responses of activated T cells. It was withdrawn in 2009 based on a report by Wroblewski *et al*[38] according to which four of 39 patients developed solid malignant tumor while on daclizumab over a follow up period of 11 years. Side effects include poor wound healing, unusual growths/lumps, swollen glands (*e.g.*, on the neck, in the armpits), unexplained weight loss, night sweats, easy bruising/bleeding, abdominal pain/swelling, unusual tiredness. A very serious allergic reaction to this drug is rare.

Anakinra: Anakinra is a recombinant non-glycosylated homologue of HuIL1Ra, a natural immunomodulating molecule, which competitively inhibits binding of IL1α and IL1β to the IL1 receptor type 1. Side effects are infections, nausea or diarrhea, headache, sinus infection, or redness, bruising, pain, or swelling at the injection site.

Tocilizumab: Tocilizumab is a recombinant humanized monoclonal antibody and inhibits IL-6 mediated responses by binding to both membrane-bound and soluble IL-6 receptors with high affinity.

Secukinumab: Secukinumab is a fully humanized IgG1k monoclonal antibody neutralizing IL-17A.

Sadreddini *et al*[39] reported treating a patient with visual loss due to retinal vasculitis resistant to prednisolone and azathioprine with rituximab successfully with a sustained remission of 24 mo of follow-up. Severe retinal vasculitis is a potentially blinding complication of patients with systemic lupus erythematosus (SLE). Hickman *et al*[40] first reported that rituximab can be used to treat severe bilateral SLE-associated retinal vasculitis. This case suggested that rituximab-induced B-cell depletion may provide an important new therapeutic option in such refractory cases. A study was conducted to evaluate the efficacy of rituximab in patients with retinal vasculitis and edema, resistant to cytotoxic drugs. Twenty patients were randomized to a rituximab group or cytotoxic combination therapy group. Rituximab was given in two 1000-mg courses (15-d interval). Subjects received methotrexate (15 mg/weekly) with prednisolone (0.5 mg/kg per day). The cytotoxic combination therapy group received pulse cyclophosphamide (1000 mg/monthly), azathioprine (2-3 mg/kg per day) and prednisolone (0.5 mg/kg per day). It was concluded that rituximab was efficient in severe ocular manifestations of Behcet's disease as significant improvement after 6 mo was seen with rituximab, but not with cytotoxic drugs[41]. A pilot study aimed to evaluate the safety, pharmacokinetics and clinical activity of gevokizumab in Behçet's disease patients with uveitis was conducted. Patients with acute posterior or panuveitis and/or retinal vasculitis, receiving 10 mg/d or less of prednisolone and resistant to azathioprine and/or cyclosporin were enrolled into the study. Patients received a single infusion of gevokizumab (0.3 mg/kg) and immunosuppressive agents were discontinued at baseline. On evaluation of the safety and uveitis status and pharmacokinetics of gevokizumab, it was found that no treatment-related adverse event was observed and rapid and durable clinical response was seen in all patients. Complete resolution of intraocular inflammation was achieved in 4-21 d, with a median duration of response of 49 d. Moreover, despite discontinuation of immunosuppressive agents and without the need to increase corticosteroid dosages, the effect was observed[42]. In addition, a clinical trial is underway for the use of anakinra in Behcet's disease (clinical trial reference number NCT01441076). Muselier *et al*[43] showed tocilizumab to be effective in treatment of refractory uveitis. Secukinumab has proved to be quite effective in the treatment of patients with anterior and posterior uveitis with no serious adverse effects[44].

Interferons

(1) IFN α; (2) Recombinant IFN α-2a (Roferon-A); (3) Recombinant IFN α-2b (Intron A); and (4) Pegylated interferons.

Interferon-α: Interferon α-2A and Interferon α-2B are human recombinant interferons manufactured using recombinant DNA technology with *E. coli* to produce human proteins. It is a type I interferon and has been used in the treatment of uveitis due to its antiproliferative, anti-angiogenic, apoptotic effects and the ability to activate dendritic, cytolytic T and natural killer cells. A prospective, open clinical trial was conducted to study long term effects of interferon α-2A on panuveitis in seven patients with Behçet's disease. IFN α-2A was given for a mean duration of 23.6 mo in seven patients. Initial dose of IFN α-2A was 6×10^6 IU/d, followed by 3×10^6 IU/d after 1 mo and 3×10^6 IU every other day after 3 mo. Additionally in the beginning of the therapy, two patients received low dose prednisolone (between 0.2 and 0.4 mg/kg per body weight). In three patients complete cessation of IFN α-2A was possible (observation period was 22, 6, and 4 mo). Six patients who had ocular manifestations of Behçet's disease for the first time or with minor damage during their course of chronic relapsing panuveitis showed marked improvement. New relapses were prevented in one patient with advanced ocular Behçet's disease. Resolution of retinal infiltrates occured within 2 wk and retinal vasculitis within 4 wk. It was found that complete remission of retinal vasculitis occurred in all patients treated with IFN α-2A alone or in combination with low dose steroids. It was concluded that retinal or optic nerve damage due to vascular occlu-

sion can be prevented by treatment with IFN α-2A. No severe side effects were found[45]. Evaluation of the efficacy of interferon needs to be done in other etiologies of retinal vasculitis through randomized studies[46,47]. Another study was conducted to evaluate the long-term development of visual acuity in patients with severe ocular Behcet's disease who were treated with IFN α-2A. Fifteen eyes of 9 patients with an active panuveitis and/or retinal vasculitis due to Behcet's disease refractory to immunosuppressive treatment were included. Visual acuity before initiation of IFN was compared to visual acuity at the end of the follow-up. Increase in visual acuity of two lines or more was seen in 10 eyes during the follow-up. In 5 eyes visual acuity remained stable. No decrease of visual acuity in any eye was seen. In the presence of macular edema, quick response to IFN α-2A was seen. It was concluded that IFN α-2A seems to be much more effective to prevent a loss or decrease of visual acuity over a long period of time in patients with severe ocular Behcet's disease compared to conventional immunosuppressants[48].

Fusion protein of cytotoxic T- lymphocyte antigen 4

Abatacept: It is a fusion protein that prevents activation of T cells by barring antigen presenting cells from delivering the co-stimulatory signals. There are case reports and case control studies reporting on the effectiveness of abatacept in the treatment of refractory uveitis in patients with juvenile idiopathic arthritis[49].

IMPORTANT CONSIDERATION

These drugs are contraindicated in patients with tuberculosis or any active infection and in patients with pregnancy or breast feeding. Patients should be instructed to avoid pregnancy till 5 mo after stopping last dose of biologics. Before prescribing them, malignant conditions should be ruled out. Baseline blood counts, liver function tests and Glucose should be measured and subsequently at every 4 wk for three months followed by every 6 wk. If patient develops fever, sore throat or bleeding then examination by a physician needs to be done. Demyelinating diseases should be ruled out before starting these drugs as TNF-α agents can aggravate multiple sclerosis. Caution should taken as reduced immunity can lead to increased risk of infection including flare up of latent tuberculosis. Also, worsening of heart failure can occur if already present.

LIMITATIONS

There is no proven causal relationship as yet with any of these novel biomarkers, though there is association of these biomarkers with some specific uveitis entities. Whether it is the disease leading to release of a specific biomarker or is it the inflammatory cytokine causing the disease is yet to be determined in future research. In addition, biologic response modifiers are expensive and with life threatening risks. Hence, a specialist experienced with immunology and the pathophysiology of inflammatory diseases has to supervise. Strict monitoring with awareness of the adverse effects is needed in rendering this specific therapy in refractory uveitis patients.

CONCLUSION

With the advent of experimental and cellular biology, cytokines are increasingly being recognized as biological markers in intraocular inflammatory diseases. Several experimental models and improved molecular biologic techniques have supported it. Biologics provide customized ocular therapy. As shown by various studies and randomized controlled trials, they have been found to be effective in several systemic diseases. Many biologic agents have been found to be efficacious in refractory anterior and posterior uveitis, particularly Behcet's disease. With the advent of novel and advanced sophisticated techniques, newer cytokines are being found. The efficacy of biologic therapies and their comparison with each other are being studied in various randomized controlled trials. In future, evidence based medicine will pave way for tailored treatment by specific biologic regime.

REFERENCES

1 **Gritz DC**, Wong IG. Incidence and prevalence of uveitis in Northern California; the Northern California Epidemiology of Uveitis Study. *Ophthalmology* 2004; **111**: 491-500; discussion 500 [PMID: 15019324 DOI: 10.1016/j.ophtha.2003.06.014]
2 **Caspi R**. Autoimmunity in the immune privileged eye: pathogenic and regulatory T cells. *Immunol Res* 2008; **42**: 41-50 [PMID: 18629448 DOI: 10.1007/s12026-008-8031-3]
3 **Luger D**, Caspi RR. New perspectives on effector mechanisms in uveitis. *Semin Immunopathol* 2008; **30**: 135-143 [PMID: 18317764 DOI: 10.1007/s00281-008-0108-5]
4 **Dick AD**. Immune mechanisms of uveitis: insights into disease pathogenesis and treatment. *Int Ophthalmol Clin* 2000; **40**: 1-18 [PMID: 10791254 DOI: 10.1097/00004397-200004000-00003]
5 **Dick AD**, Carter DA. Cytokines and immunopathogenesis of intraocular posterior segment inflammation. *Ocul Immunol Inflamm* 2003; **11**: 17-28 [PMID: 12854024 DOI: 10.1076/ocii.11.1.17.15575]
6 **Abbas AK**, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature* 1996; **383**: 787-793 [PMID: 8893001]
7 **Sakaguchi S**. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004; **22**: 531-562 [PMID: 15032588 DOI: 10.1002/ijc.25429]
8 **Korn T**, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol* 2009; **27**: 485-517 [PMID: 19132915 DOI: 10.1146/annurev.immunol.021908.132710]
9 **Akdis M**. The cellular orchestra in skin allergy; are differences to lung and nose relevant? *Curr Opin Allergy Clin Immunol* 2010; **10**: 443-451 [PMID: 20736733 DOI: 10.1097/ACI.0b013e32833d7d48]
10 **Ooi KG**, Galatowicz G, Calder VL, Lightman SL. Cytokines and chemokines in uveitis: is there a correlation with clinical phenotype? *Clin Med Res* 2006; **4**: 294-309 [PMID: 17210978 DOI: 10.3121/cmr.4.4.294]
11 **Saxena S**, Pant AB, Khanna VK, Singh K, Shukla RK, Meyer

CH, Singh VK. Tumor necrosis factor-α-mediated severity of idiopathic retinal periphlebitis in young adults (Eales' disease): implication for anti-TNF-α therapy. *J Ocul Biol Dis Infor* 2010; **3**: 35-38 [PMID: 21139707 DOI: 10.1007/s12177-010-9053-3]

12 **Saxena S**, Khanna VK, Pant AB, Meyer CH, Singh VK. Elevated tumor necrosis factor in serum is associated with increased retinal ischemia in proliferative eales' disease. *Pathobiology* 2011; **78**: 261-265 [PMID: 21849807 DOI: 10.1159/000329589]

13 **Saxena S**, Pant AB, Khanna VK, Agarwal AK, Singh K, Kumar D, Singh VK. Interleukin-1 and tumor necrosis factor-alpha: novel targets for immunotherapy in Eales disease. *Ocul Immunol Inflamm* 2009; **17**: 201-206 [PMID: 19585364 DOI: 10.1080/09273940902731015]

14 **Ahn JK**, Yu HG, Chung H, Park YG. Intraocular cytokine environment in active Behçet uveitis. *Am J Ophthalmol* 2006; **142**: 429-434 [PMID: 16935587 DOI: 10.1016/j.ajo.2006.04.016]

15 **Ooi KG**, Galatowicz G, Towler HM, Lightman SL, Calder VL. Multiplex cytokine detection versus ELISA for aqueous humor: IL-5, IL-10, and IFNgamma profiles in uveitis. *Invest Ophthalmol Vis Sci* 2006; **47**: 272-277 [PMID: 16384973]

16 **El-Asrar AM**, Struyf S, Kangave D, Al-Obeidan SS, Opdenakker G, Geboes K, Van Damme J. Cytokine profiles in aqueous humor of patients with different clinical entities of endogenous uveitis. *Clin Immunol* 2011; **139**: 177-184 [PMID: 21334264 DOI: 10.1016/j.clim.2011.01.014]

17 **Curnow SJ**, Falciani F, Durrani OM, Cheung CM, Ross EJ, Wloka K, Rauz S, Wallace GR, Salmon M, Murray PI. Multiplex bead immunoassay analysis of aqueous humor reveals distinct cytokine profiles in uveitis. *Invest Ophthalmol Vis Sci* 2005; **46**: 4251-4259 [PMID: 16249505 DOI: 10.1167/iovs.05-0444]

18 **Atan D**, Fraser-Bell S, Plskova J, Kuffova L, Hogan A, Tufail A, Kilmartin DJ, Forrester JV, Bidwell J, Dick AD, Churchill AJ. Cytokine polymorphism in noninfectious uveitis. *Invest Ophthalmol Vis Sci* 2010; **51**: 4133-4142 [PMID: 20335604 DOI: 10.1167/iovs.09-4583]

19 **Takeuchi H**, Remington G. A systematic review of reported cases involving psychotic symptoms worsened by aripiprazole in schizophrenia or schizoaffective disorder. *Psychopharmacology* (Berl) 2013; **228**: 175-185 [PMID: 23736279 DOI: 10.1007/s00213-013-3154-1]

20 **Dick AD**. Experimental approaches to specific immunotherapies in autoimmune disease: future treatment of endogenous posterior uveitis? *Br J Ophthalmol* 1995; **79**: 81-88 [PMID: 7880799 DOI: 10.1136/bjo.79.1.81]

21 **Imrie FR**, Dick AD. Biologics in the treatment of uveitis. *Curr Opin Ophthalmol* 2007; **18**: 481-486 [PMID: 18163000 DOI: 10.1097/ICU.0b013e3282f03d42]

22 **Sharma SM**, Dick AD, Ramanan AV. Non-infectious pediatric uveitis: an update on immunomodulatory management. *Paediatr Drugs* 2009; **11**: 229-241 [PMID: 19566107 DOI: 10.2165/00148581-200911040-00002]

23 **Willermain F**, Rosenbaum JT, Bodaghi B, Rosenzweig HL, Childers S, Behrend T, Wildner G, Dick AD. Interplay between innate and adaptive immunity in the development of non-infectious uveitis. *Prog Retin Eye Res* 2012; **31**: 182-194 [PMID: 22120610 DOI: 10.1016/j.preteyeres.2011.11.004]

24 **Bali T**, Saxena S, Kumar D, Nath R. Response time and safety profile of pulsed oral methotrexate therapy in idiopathic retinal periphlebitis. *Eur J Ophthalmol* 2005; **15**: 374-378 [PMID: 15945007]

25 **Saxena S**. Combined oral corticosteroid-methotrexate therapy in Eales' disease. *Ann Ophthalmol* (Skokie) 2009; **41**: 93-97 [PMID: 19845224]

26 **Takeuchi M**. A systematic review of biologics for the treatment of noninfectious uveitis. *Immunotherapy* 2013; **5**: 91-102 [PMID: 23256801 DOI: 10.2217/imt.12.134]

27 **De Rycke L**, Baeten D, Kruithof E, Van den Bosch F, Veys EM, De Keyser F. Infliximab, but not etanercept, induces IgM anti-double-stranded DNA autoantibodies as main antinuclear reactivity: biologic and clinical implications in autoimmune arthritis. *Arthritis Rheum* 2005; **52**: 2192-2201 [PMID: 15986349 DOI: 10.1002/art.21190]

28 **Iwata D**, Namba K, Mizuuchi K, Kitaichi N, Kase S, Takemoto Y, Ohno S, Ishida S. Correlation between elevation of serum antinuclear antibody titer and decreased therapeutic efficacy in the treatment of Behçet's disease with infliximab. *Graefes Arch Clin Exp Ophthalmol* 2012; **250**: 1081-1087 [PMID: 22234352 DOI: 10.1007/s00417-011-1908-1]

29 **Markomichelakis N**, Delicha E, Masselos S, Fragiadaki K, Kaklamanis P, Sfikakis PP. A single infliximab infusion vs corticosteroids for acute panuveitis attacks in Behçet's disease: a comparative 4-week study. *Rheumatology* (Oxford) 2011; **50**: 593-597 [PMID: 21097877 DOI: 10.1093/rheumatology/keq366]

30 **Petropoulos IK**, Vaudaux JD, Guex-Crosier Y. Anti-TNF-alpha therapy in patients with chronic non-infectious uveitis: the experience of Jules Gonin Eye Hospital. *Klin Monbl Augenheilkd* 2008; **225**: 457-461 [PMID: 18454398 DOI: 10.1055/s-2008-1027361]

31 **Cruz BA**, Reis DD, Araujo CA. Refractory retinal vasculitis due to sarcoidosis successfully treated with infliximab. *Rheumatol Int* 2007; **27**: 1181-1183 [PMID: 17520259 DOI: 10.1097/01.rhu.0000217187.70246.2b]

32 **Rajaraman RT**, Kimura Y, Li S, Haines K, Chu DS. Retrospective case review of pediatric patients with uveitis treated with infliximab. *Ophthalmology* 2006; **113**: 308-314 [PMID: 16406545 DOI: 10.1016/j.ophtha.2005.09.037]

33 **Olivieri I**, Leccese P, D'Angelo S, Padula A, Nigro A, Palazzi C, Coniglio G, Latanza L. Efficacy of adalimumab in patients with Behçet's disease unsuccessfully treated with infliximab. *Clin Exp Rheumatol* 2011; **29**: S54-S57 [PMID: 21968237]

34 **Evereklioglu C**. Ocular Behçet disease: current therapeutic approaches. *Curr Opin Ophthalmol* 2011; **22**: 508-516 [PMID: 21897239 DOI: 10.1016/j.survophthal.2005.04.009]

35 **Rifkin LM**, Birnbaum AD, Goldstein DA. TNF inhibition for ophthalmic indications: current status and outlook. *BioDrugs* 2013; **27**: 347-357 [PMID: 23568177 DOI: 10.1007/s40259-013-0022-9]

36 **Mesquida M**, Victoria Hernández M, Llorenç V, Pelegrín L, Espinosa G, Dick AD, Adán A. Behçet disease-associated uveitis successfully treated with golimumab. *Ocul Immunol Inflamm* 2013; **21**: 160-162 [PMID: 23252659 DOI: 10.3109/09273948.2012.741744]

37 **William M**, Faez S, Papaliodis GN, Lobo AM. Golimumab for the treatment of refractory juvenile idiopathic arthritis-associated uveitis. *J Ophthalmic Inflamm Infect* 2012; **2**: 231-233 [PMID: 22581347 DOI: 10.1007/s12348-012-0081-y]

38 **Wroblewski K**, Sen HN, Yeh S, Faia L, Li Z, Sran P, Gangaputra S, Vitale S, Sherry P, Nussenblatt R. Long-term daclizumab therapy for the treatment of noninfectious ocular inflammatory disease. *Can J Ophthalmol* 2011; **46**: 322-328 [PMID: 21816251 DOI: 10.1016/j.jcjo.2011.06.008]

39 **Sadreddini S**, Noshad H, Molaeefard M, Noshad R. Treatment of retinal vasculitis in Behçet's disease with rituximab. *Mod Rheumatol* 2008; **18**: 306-308 [PMID: 18438602 DOI: 10.3109/s10165-008-0057-9]

40 **Hickman RA**, Denniston AK, Yee CS, Toescu V, Murray PI, Gordon C. Bilateral retinal vasculitis in a patient with systemic lupus erythematosus and its remission with rituximab therapy. *Lupus* 2010; **19**: 327-329 [PMID: 19900982 DOI: 10.1177/0961203309347332]

41 **Davatchi F**, Shams H, Rezaipoor M, Sadeghi-Abdollahi B, Shahram F, Nadji A, Chams-Davatchi C, Akhlaghi M, Faezi T, Naderi N. Rituximab in intractable ocular lesions of Behcet's disease; randomized single-blind control study (pilot study). *Int J Rheum Dis* 2010; **13**: 246-252 [PMID: 20704622 DOI: 10.1111/j.1756-185X.2010.01546.x]

42 **Gül A**, Tugal-Tutkun I, Dinarello CA, Reznikov L, Esen BA, Mirza A, Scannon P, Solinger A. Interleukin-1β-regulating antibody XOMA 052 (gevokizumab) in the treatment of acute exacerbations of resistant uveitis of Behcet's disease: an open-label pilot study. *Ann Rheum Dis* 2012; **71**: 563-566 [PMID: 22084392 DOI: 10.1136/annrheumdis-2011-155143]

43 **Muselier A**, Bielefeld P, Bidot S, Vinit J, Besancenot JF, Bron A. Efficacy of tocilizumab in two patients with anti-TNF-alpha refractory uveitis. *Ocul Immunol Inflamm* 2011; **19**: 382-383 [PMID: 21970668 DOI: 10.3109/09273948.2011.606593]

44 **Hueber W**, Patel DD, Dryja T, Wright AM, Koroleva I, Bruin G, Antoni C, Draelos Z, Gold MH, Durez P, Tak PP, Gomez-Reino JJ, Foster CS, Kim RY, Samson CM, Falk NS, Chu DS, Callanan D, Nguyen QD, Rose K, Haider A, Di Padova F. Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. *Sci Transl Med* 2010; **2**: 52ra72 [PMID: 20926833 DOI: 10.1126/scitranslmed.3001107]

45 **Kötter I**, Eckstein AK, Stübiger N, Zierhut M. Treatment of ocular symptoms of Behçet's disease with interferon alpha 2a: a pilot study. *Br J Ophthalmol* 1998; **82**: 488-494 [PMID: 9713053 DOI: 10.1136/bjo.87.4.423]

46 **Fardeau C**. [Interferon and retinal vasculitis]. *J Fr Ophtalmol* 2006; **29**: 392-397 [PMID: 16885805 DOI: 10.1016/S0181-5512(06)77697-5]

47 **Kötter I**, Zierhut M, Eckstein A, Vonthein R, Ness T, Günaydin I, Grimbacher B, Blaschke S, Peter HH, Kanz L, Stübiger N. Human recombinant interferon-alpha2a (rhIFN alpha2a) for the treatment of Behçet's disease with sight-threatening retinal vasculitis. *Adv Exp Med Biol* 2003; **528**: 521-523 [PMID: 12918755 DOI: 10.1007/0-306-48382-3_104]

48 **Deuter CM**, Kötter I, Günaydin I, Zierhut M, Stübiger N. [Ocular involvement in Behçet's disease: first 5-year-results for visual development after treatment with interferon alfa-2a]. *Ophthalmologe* 2004; **101**: 129-134 [PMID: 14991308 DOI: 10.1007/s00347-003-0927-7]

49 **Kenawy N**, Cleary G, Mewar D, Beare N, Chandna A, Pearce I. Abatacept: a potential therapy in refractory cases of juvenile idiopathic arthritis-associated uveitis. *Graefes Arch Clin Exp Ophthalmol* 2011; **249**: 297-300 [PMID: 20922440 DOI: 10.1007/s00417-010-1523-6]

P- Reviewer: Issa SA, Machida S **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Wang CH

World Journal of Immunology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
www.wjgnet.com

World J Immunol 2014 July 27; 4(2): I-V
ISSN 2219-2824 (online)

INSTRUCTIONS TO AUTHORS

GENERAL INFORMATION

World Journal of Immunology (*World J Immunol*, *WJI*, online ISSN 2219-2824, DOI: 10.5411) is a peer-reviewed open access (OA) academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

Aims and scope

WJI covers a wide range of subjects including: (1) autoimmune diseases such as type 1 diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, thyroiditis, myasthenia gravis, in both humans and animal models of disease, with an interest on aspects including the etiology, pathogenesis, mechanisms of disease induction, maintenance and progression; (2) tumor immunology including immunosurveillance, immunoediting and immunotherapies in animal models and in humans; (3) clinical immunology in humans and animal models including mechanisms of disease, regulation and therapy and immunodeficiencies; (4) innate immunity including cell subsets, receptors and soluble mediators, complement and inflammation; (5) adaptive immune mechanisms and cells including soluble mediators and antibodies; (6) immune cell development, differentiation, maturation; (7) control mechanisms for immune cells including immune tolerance and apoptosis; (8) immune cell interactions and immune cell receptors; (9) immunological methods and techniques; (10) immune cell activation including cell signaling pathways, biochemical and pharmacologic modulation studies; (11) infection; (12) different modalities of vaccination including gene therapy; (13) hypersensitivity and allergy; (14) transplantation.

We encourage authors to submit their manuscripts to *WJI*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

WJI is edited and published by Baishideng Publishing Group (BPG). BPG has a strong professional editorial team composed of science editors, language editors and electronic editors. BPG currently publishes 43 OA clinical medical journals, including 42 in English, has a total of 15471 editorial board members or peer reviewers, and is a world first-class publisher

Columns

The columns in the issues of *WJI* will include: (1) Editorial: The editorial board members are invited to make comments on an important topic in their field in terms of its current research status and future directions to lead the development of this discipline; (2) Frontier: The editorial board members are invited to select a highly cited cutting-edge original paper of his/her own to summarize major findings, the problems that have been resolved and remain to be resolved, and future research directions to help readers understand his/her important academic point of view and future research directions in the field; (3) Diagnostic Advances: The editorial board members are invited to write high-quality diagnostic advances in their field to improve the diagnostic skills of readers. The topic covers general clinical diagnosis, differential diagnosis, pathological diagnosis, laboratory diagnosis, imaging diagnosis, endoscopic diagnosis, biotechnological diagnosis, functional diagnosis, and physical diagnosis; (4) Therapeutics Advances: The editorial board members are invited to write high-quality therapeutic advances in their field to help improve the therapeutic skills of readers. The topic covers medication therapy, psychotherapy, physical therapy, replacement therapy, interventional therapy, minimally invasive therapy, endoscopic therapy, transplantation therapy, and surgical therapy; (5) Field of Vision: The editorial board members are invited to write commentaries on classic articles, hot topic articles, or latest articles to keep readers at the forefront of research and increase their levels of clinical research. Classic articles refer to papers that are included in Web of Knowledge and have received a large number of citations (ranking in the top 1%) after being published for more than years, reflecting the quality and impact of papers. Hot topic articles refer to papers that are included in Web of Knowledge and have received a large number of citations after being published for no more than 2 years, reflecting cutting-edge trends in scientific research. Latest articles refer to the latest published high-quality papers that are included in PubMed, reflecting the latest research trends. These commentary articles should focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions. Basic information about the article to be commented (including authors, article title, journal name, year, volume, and inclusive page numbers; (6) Minireviews: The editorial board members are invited to write short reviews on recent advances and trends in research of molecular biology, genomics, and related cutting-edge technologies to provide readers with the latest knowledge and help improve their diagnostic and therapeutic skills; (7) Review: To make a systematic review to focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions; (8) Topic Highlight: The editorial board members are invited to write a series of articles (7-10 articles) to comment and discuss a hot topic to help improve the diagnostic and therapeutic skills of readers; (9) Medical Ethics: The editorial board members are invited to write articles about medical ethics to increase readers' knowledge of medical ethics. The topic covers international ethics guidelines, animal studies, clinical trials, organ transplantation, *etc.*; (10) Clinical Case Conference or Clinicopathological Conference: The editorial board members are invited to contribute high-quality clinical case conference; (11) Original Articles: To report innovative and original findings in immunology; (12) Research Report: To briefly report the novel and innovative findings in immunology; (13) Meta-Analysis: To summarize a given quantitative effect, *e.g.*, the clinical effectiveness and safety of clinical treatments by combining data from two or more randomized controlled trials, thereby providing more precise and externally valid estimates than those which would stem from each individual dataset if analyzed separately from the others; (14) Case Report: To report a rare or typical case; (15) Letters to the Editor: To discuss and make reply to the contributions published in *WJI*, or to introduce and comment on a controversial issue of general interest; (16) Book Reviews: To introduce and comment on quality monographs of immunology; and (17) Autobiography: The editorial board members are invited to write their autobiography to provide readers with stories of success or failure in their scientific research career. The topic covers their basic personal information and information about when they started doing research work, where and how they did research work, what they have achieved, and their lessons from success or failure.

Name of journal

World Journal of Immunology

ISSN
ISSN 2219-2824 (online)

Launch date
December 27, 2011

Frequency
Four-monthly

Editorial office
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Gastrointestinal Surgery
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

Publisher
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

Instructions to authors
Full instructions are available online at http://www.wjgnet.com/2219-2824/g_info_20100316161927.htm.

Indexed and Abstracted in
Digital Object Identifier.

SPECIAL STATEMENT

All articles published in journals owned by the BPG represent the views and opinions of their authors, and not the views, opinions or policies of the BPG, except where otherwise explicitly indicated.

Biostatistical editing
Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

Conflict-of-interest statement
In the interests of transparency and to help reviewers assess any potential bias, *WJI* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

Sample wording: [Name of individual] has received fees for serving as a speaker, a consultant and an advisory board member for [names of organizations], and has received research funding from [names of organization]. [Name of individual] is an employee of [name of organization]. [Name of individual] owns stocks and shares in [name of organization]. [Name of individual] owns patent [patent identification and brief description].

Statement of informed consent
Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

Statement of human and animal rights
When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of BPG, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry

at its outset. The only register now available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://www.wjgnet.com/esps/. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/2219-2824/g_info_20100722180909.htm) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to bpgoffice@wjgnet.com, or by telephone: +86-10-85381891. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Title: Title should be less than 12 words.

Running title: A short running title of less than 6 words should be provided.

Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

Institution: Author names should be given first, then the complete name of institution, city, province and postcode. For example, Xu-Chen Zhang, Li-Xin Mei, Department of Pathology, Chengde Medical College, Chengde 067000, Hebei Province, China. One author may be represented from two institutions, for example, George Sgourakis, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany; George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece

Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

Supportive foundations: The complete name and number of supportive foundations should be provided, e.g. Supported by National Natural Science Foundation of China, No. 30224801

Correspondence to: Only one corresponding address should be provided. Author names should be given first, then author title, affiliation, the complete name of institution, city, postcode, province, country, and email. All the letters in the email should be in lower case. A space interval should be inserted between country name and email address. For example, Montgomery Bissell, MD, Professor of Medicine, Chief, Liver Center, Gastroenterology Division, University of California, Box 0538, San Francisco, CA 94143, United States. montgomery.bissell@ucsf.edu

Telephone and fax: Telephone and fax should consist of +, country number, district number and telephone or fax number, e.g. Telephone: +86-10-85381892 Fax: +86-10-85381893

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision on acceptance is made only when at least two experts recommend publication of an article. All peer-reviewers are acknowledged on Express Submission and Peer-review System website.

Abstract

There are unstructured abstracts (no less than 200 words) and structured abstracts. The specific requirements for structured abstracts are as follows:

An informative, structured abstract should accompany each manuscript. Abstracts of original contributions should be structured into the following sections: AIM (no more than 20 words; Only the purpose of the study should be included. Please write the Aim in the form of "To investigate/study/…"), METHODS (no less than 140 words for Original Articles; and no less than 80 words for Brief Articles), RESULTS (no less than 150 words for Original Articles and no less than 120 words for Brief Articles; You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, *e.g.*, 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$), and CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Core tip

Please write a summary of less than 100 words to outline the most innovative and important arguments and core contents in your paper to attract readers.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a

second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID and DOI

Pleased provide PubMed citation numbers to the reference list, e.g. PMID and DOI, which can be found at http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed and http://www.crossref.org/SimpleTextQuery/, respectively. The numbers will be used in E-version of this journal.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool

assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data
Write as mean ± SD or mean ± SE.

Statistical expression
Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units
Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23243641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/2219-2824/g_info_20100725073806.htm.

Abbreviations
Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics
Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

Examples for paper writing
All types of articles' writing style and requirement will be found in the link: http://www.wjgnet.com/esps/NavigationInfo.aspx?id=15

RESUBMISSION OF THE REVISED MANUSCRIPTS
Authors must revise their manuscript carefully according to the revision policies of BPG. The revised version, along with the signed copyright transfer agreement, responses to the reviewers, and English language Grade A certificate (for non-native speakers of English), should be submitted to the online system *via* the link contained in the e-mail sent by the editor. If you have any questions about the revision, please send e-mail to esps@wjgnet.com.

Language evaluation
The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; and (4) Grade D: rejected. Revised articles should reach Grade A.

Copyright assignment form
Please download a Copyright assignment form from http://www.wjgnet.com/2219-2824/g_info_20100725073806.htm.

Responses to reviewers
Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/2219-2824/g_info_20100725073806.htm.

Proof of financial support
For papers supported by a foundation, authors should provide a copy of the approval document and serial number of the foundation.

STATEMENT ABOUT ANONYMOUS PUBLICATION OF THE PEER REVIEWERS' COMMENTS
In order to increase the quality of peer review, push authors to carefully revise their manuscripts based on the peer reviewers' comments, and promote academic interactions among peer reviewers, authors and readers, we decide to anonymously publish the reviewers' comments and author's responses at the same time the manuscript is published online.

PUBLICATION FEE
WJI is an international, peer-reviewed, OA online journal. Articles published by this journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium and format, provided the original work is properly cited. The use is non-commercial and is otherwise in compliance with the license. Authors of accepted articles must pay a publication fee. Publication fee: 698 USD per article. All invited articles are published free of charge.

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com