ISSN 2219-2824 (online)

World Journal of *Immunology*

World J Immunol 2014 March 27; 4(1): 1-41

Baishideng Publishing Group
www.wjgnet.com

WJI

World Journal of Immunology

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

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2011-2015

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WJI

World Journal of Immunology

Contents

Four-monthly Volume 4 Number 1 March 27, 2014

World Journal of Immunology
Volume 4 Number 1 March 27, 2014

Contents

NAME OF JOURNAL
World Journal of Immunology

ISSN
ISSN 2219-2824 (online)

LAUNCH DATE
December 27, 2011

FREQUENCY
Four-monthly

EDITORIAL OFFICE
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PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza,
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Hong Kong, China
Telephone: +852-6555-7188
Fax: +852-3177-9906
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http://www.wjgnet.com

PUBLICATION DATE
March 27, 2014

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/2219-2824/g_info_20100722180909.htm.

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Immunology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5411/wji.v4.i1.1

World J Immunol 2014 March 27; 4(1): 1-11
ISSN 2219-2824 (online)

REVIEW

Immune response after photodynamic therapy increases anti-cancer and anti-bacterial effects

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Received: October 17, 2013 Revised: December 20, 2013
Accepted: February 16, 2014
Published online: March 27, 2014

Abstract

Photodynamic therapy (PDT) is a clinically approved procedure for treatment of cancer and infections. PDT involves systemic or topical administration of a photosensitizer (PS), followed by irradiation of the diseased area with light of a wavelength corresponding to an absorbance band of the PS. In the presence of oxygen, a photochemical reaction is initiated, leading to the generation of reactive oxygen species and cell death. Besides causing direct cytotoxic effects on illuminated tumor cells, PDT is known to cause damage to the tumor vasculature and induce the release of pro-inflammatory molecules. Pre-clinical and clinical studies have demonstrated that PDT is capable of affecting both the innate and adaptive arms of the immune system. Immune stimulatory properties of PDT may increase its beneficial effects giving the therapy wider potential to become more extensively used in clinical practice. Besides stimulating tumor-specific cytotoxic T-cells capable to destroy distant untreated tumor cells, PDT leads to development of anti-tumor memory immunity that can potentially prevent the recurrence of cancer. The immunological effects of PDT make the therapy more effective also when used for treatment of bacterial infections, due to an augmented infiltration of neutrophils into the infected regions that seems to potentiate the outcome of the treatment.

Key words: Photodynamic therapy; Anti-tumor immunity; T-cell activation; Damage-associated molecular patterns; Inflammatory cells

Core tip: The immune stimulatory properties of photodynamic therapy (PDT) make this therapy one of the most promising therapeutic procedures for the management of cancer lesions and microbial infections. This review will focus on the current knowledge of the innate and adaptive immune responses induced by PDT against tumors and pathogens.

Reginato E, Wolf P, Hamblin MR. Immune response after photodynamic therapy increases anti-cancer and anti-bacterial effects. *World J Immunol* 2014; 4(1): 1-11 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i1/1.htm DOI: http://dx.doi.org/10.5411/wji.v4.i1.1

INTRODUCTION

The ideal therapy for cancer should be able to selectively destroy the tumor cells at the primary site and at the same time trigger the immune system to recognize any remaining or recurring cancer cells. Compared to other unspecific and/or immunosuppressive cancer therapies

such as chemotherapy, ionizing radiation and surgery, photodynamic therapy (PDT) might have these desirable properties. PDT is a procedure that consists of three components: A photosensitizer (PS), light of appropriate wavelength to excite the PS and molecular oxygen[1,2]. None of these three components is individually toxic, but when combined together they initiate a photochemical reaction that culminates in the generation of highly reactive oxygen species (ROS)[3]. Most of the PSs used in PDT are based on a tetrapyrrole structure, similar to that of the protoporphyrin contained in hemoglobin[4]. They have an absorption peak between 600 and 800 nm (red to deep red), since light at lower wavelengths would not penetrate efficiently through the tissue and light at longer wavelengths than 800 nm would not have sufficient energy to initiate a photochemical reaction and generate a substantial yield of ROS[4].

The ROS produced during PDT can directly kill tumor cells by induction of necrosis and/or apoptosis[5] and damage the tumor vasculature, leading to depletion of oxygen and nutrients in the tumor[6,7]. As a result of this traumatic insult to the tumor and its microenvironment, a strong acute inflammatory reaction is provoked at the targeted site[1]. The acute inflammatory response following PDT causes infiltration of host innate immune cells that carry out the removal of damaged cells. Acute inflammation also seems to be implicated in the development of adaptive anti-tumor immunity[1]. In particular, the efficacy of PDT in some models has been shown to be dependent upon such induction of anti-tumor immunity. Early studies showed that while PDT of EMT6 tumors exhibited curative effects and long-term tumor control in Balb/c mice, the long-term protection from tumors was lost when PDT was performed in either *scid* (which lack T and B cells), or nude (which lack T cells) immune-compromised mice. However, when the *scid* mice were reconstituted with splenic T cells or bone marrow cells from Balb/c mice, the curative effect of PDT was restored[8,9].

While the immune stimulatory effects of PDT have been widely studied, although not completely understood in cancer models, much effort still has to be done to understand these effects of PDT in microbial infections. Tanaka *et al*[10] discovered that the therapeutic effect of PDT in a mouse model of bacterial arthritis was dependent on the attraction and accumulation of neutrophils into the infected region and could also produce a protective effect if carried out before infection. This review will focus on the current knowledge of the beneficial immunological effects of PDT for cancer and bacterial infections. A list of notable publications that show that PDT can activate different constituents of the immune system is provided in Table 1.

DAMAGE-ASSOCIATED MOLECULAR PATTERNS

After the traumatic insult to the tumor induced by PDT, one of the first events occurring at the treatment site is the generation of "danger" signals, so called damage-associated molecular patterns (DAMPs) or cell death-associated molecular patterns (CDAMPs) that serve as warning signals in innate immunity[11-14]. DAMPs play a similar role to that of pathogen-associated molecular patterns, but instead of being associated with pathogenic microbes, they are associated with host tissue damage. DAMPs are endogenous intracellular molecules normally "hidden" within living cells, but upon exposure or secretion from dying and/or damaged cells, they acquire immune-stimulatory properties. DAMPs are thought to be the key mediators of the immunogenicity of tumor cells killed by PDT *via* necrosis or apoptosis. They constitute alarm signals warning that "self-altered" antigens were released from dying cells; the immune system recognizes them and triggers a vigorous immunological response. It is generally accepted that while necrotic cells are pro-inflammatory and immunogenic, some forms of apoptotic cells are efficiently engulfed and disposed of by macrophages and other phagocytic cells, therefore they should not induce inflammation and are unlikely to stimulate the immune system[15,16]. However, it has been reported that under certain circumstances, other forms of apoptotic cells such as tumor cells undergoing apoptosis by some particular cancer therapies can effectively generate an immune response[17,18]. In this case the process is defined as "immunogenic apoptosis" *vs* the conventional "non-immunogenic apoptosis"[16,19,20].

It is conceivable that while the physiological programmed cell death is non-inflammatory and non-immunogenic, some cancer therapies (such as particular forms of chemotherapy and PDT) cause tumor damage, and produce an immunogenic form of apoptosis characterized by release of DAMPs and enhancement of inflammation.

The release of DAMPs after PDT has been investigated in some studies[11,12,21]. Korbelik *et al*[22] found that squamous cell carcinoma VII (SCCVII) cancer cells treated by *in vitro* photofrin-PDT expose on the surface heat shock proteins (HSPs) such as HSP60, HSP70 and glucose-regulated protein 94 (GRP94) and release HSP70 to the extracellular space. Interestingly, when PDT was applied in *in vivo* settings, they found a different spectrum of DAMPs exposed on the surface of treated SCCVII cells. While HSP70 was still exposed, HSP60 and GRP94 were no longer detected and replaced by GRP78 on the surface of PDT-treated SCCVII cancer cells. This indicated for the first time that the DAMPs associated with PDT can differ in the same cancer cells between *in vitro* and *in vitro* settings[22].

It is worth mentioning also that the spectra of DAMPs exposed and/or released after PDT correlate with the sub-cellular localization patterns of the PS, where the ROS-based stress is originated. For instance, PSs targeting the endoplasmatic reticulum (*e.g.*, hypericin) are known to cause surface exposure of calreticulin (CRT); conversely, Photofrin (whose localization is mostly associated with lipid membranes)-PDT, has been linked primarily to surface exposure of HSP70[23,24].

Table 1 Milestone studies on effects of photodynamic therapy affecting the immune system

Immune components	Immunomodulatory effect of PDT	Ref.
Pro-inflammatory cytokines	Production of pro-inflammatory cytokines after PDT *in vivo*	[26]
Macrophages	First evidence of cytokine production by PDT-treated macrophages *in vivo*	[107]
Dendritic cells	DCs can efficiently phagocytose PDT-treated tumor cells in *in vivo* experiments. Immature DCs administered in combination with PDT produce effective antitumor response *in vivo*	[38,108]
NKs	Role of NKs in immune response after PDT, control of distant untreated tumors	[43]
Neutrophils	Evidences that neutrophils have a crucial role in the PDT response *in vivo*	[30,109]
Memory immunity	First demonstration that a specific antitumor memory immunity is induced after PDT: resistance to tumor rechallenge in animals cured by PDT	[110]
T lymphocytes, memory immunity	Essential role of host T lymphocytes in immune response after PDT: curative effect of PDT in immune-competent Balb/c mice, but not in immune-suppressed *scid* mice. Adoptive transfer of splenocytes from PDT-cured mice to *scid* mice confers resistance to tumor rechallenge	[8,111]
Treg	Evidences for the role of Treg in inhibiting the immune response after PDT	[77]
Patient lymphocytes	First demonstration that an antigen-specific immune response can be observed after PDT	[65]

PDT: Photodynamic therapy; DCs: Dendritic cells; NKs: Natural killer cells; Treg: T regulatory cells.

Table 2 Damage-associated molecular pattern molecules that may be released or exposed on the outer leaflet of dying tumor cells after photodynamic therapy

DAMP	Function	Ref.
HSP60, HSP70, HSP90, gp96, GRP94, GRP78	Molecular chaperones that normally reside in intracellular regions/organelles, but under stress they are exposed on the damaged cell surface and prime immunomodulatory processes	[11,21,22,112]
Calreticulin	Calcium binding protein located in intracellular regions/organelles (mostly in ER), but under stress its presence on the PM is augmented. On the PM it acts as "danger signal" and increases the immunogenicity of the dying cells	[11,112]
ATP	High-energy molecule, normally intracellular, but can be released by necrotic and apoptotic cells under particular stresses. Extracellular ATP has the ability to help in chemoattraction of immune cells	[12,112]
Phosphatidylserine	When cells are damaged/dying, phosphatidylserine is transposed from the inner to the outer leaflet and acts as an "eat me" signal by interacting with multiple immune cells receptors, mediating efficient phagocytosis and anti-inflammatory responses	[112,113]
High mobility group box-1	Nuclear chromatin-binding protein; it has prominent cytokine-line properties and when released by dying cells tends to stimulate immune cells to produce various pro-inflammatory cytokines	[11,112]
Calgranulin family members (S100A8, S100A9, S100A12)	Calcium-binding proteins; when released by necrotic cells they act as "find me" signals attracting various immune cells and interacting with immune cell receptor (TLR4/RAGE) to induce the secretion of pro-inflammatory cytokines	[11,112,114]
Cross-linked dimer of ribosomal protein S19	Constituent of small ribosomal subunit; when released by necrotic cells it acts as a chemotactic factor for attracting various immune cells	[11,112]

DAMPs: Damage-associated molecular patterns; ER: Endoplasmatic reticulum; PM: Plasma membrane; HSP: Heat shock protein; GRP: Glucose-regulated protein; ATP: Adenosine triphosphate; TLR4: Toll-like receptor 4; RAGE: Receptor for advanced glycation end-products.

Further investigations on cellular and molecular mechanisms are certainly required to establish in more detail the correlations between DAMPs and PDT. However, the most important examples of DAMPs which are produced after PDT reported so far are HSPs, CRT, adenosine triphosphate and other mediators[21,22,25]. Table 2 lists the DAMPs that have been reported to be produced after PDT.

INFLAMMATION AND INNATE IMMUNE RESPONSES IN ANTI-CANCER PDT

The PDT-induced oxidative stress and traumatic insult to the tumor microenvironment are known to stimulate the release or expression of various proinflammatory mediators [tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1, complement proteins, HSPs and arachidonic acid metabolites] from the treated site[26]. Moreover, as mentioned above, immunogenic DAMPs are released after PDT and they can be detected by the innate immune cells that are programmed to detect microbial invasion[27]. For these reasons, innate immune cells such as monocytes or macrophages, neutrophils and dendritic cells (DCs) are recruited to the treated site and infiltrate in large numbers to attack what is expected to be a microbial invasion but turns out to be damaged tumor cells[28]. The primary function of the inflammatory cells is to neutralize the DAMPs by engulfing and eliminating the cellular debris as well as compromised tissue components. This promotes local healing with restoration of normal tissue function. At the onset of PDT-induced inflammation, the tumor vasculature undergoes significant changes and become permeable for blood proteins and pro-adhesive for inflammatory cells *via* over-expression of adhesion molecules (Intracellular Adhesion Molecule 1, Vascular Cell Adhesion Molecule 1, selectins)[27], thus favoring the

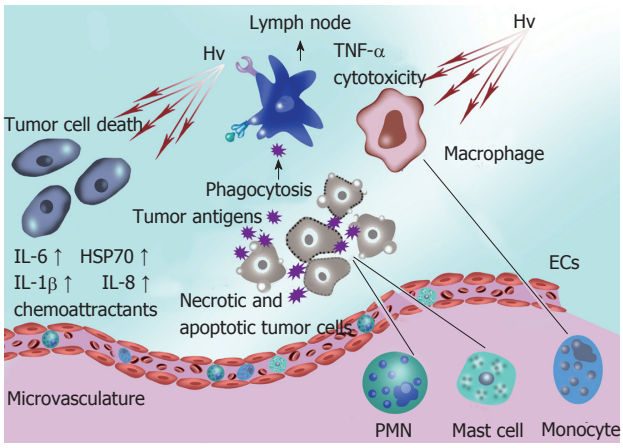

Figure 1 Innate immune responses in anti-cancer photodynamic therapy. Photodynamic therapy of tumors leads to the development of local inflammation mediated by the release of danger signals and cytokines. Various cells of the immune system infiltrate into the treated area. ECs: Endothelial cells; HSP70: Heat-shock protein; Hv: Light; PMNs: Polymorphonuclear neutrophils; TNF: Tumor necrosis factor; IL-6: Interleukin-6. Original figure based upon Ref. [115] and Ref. [116].

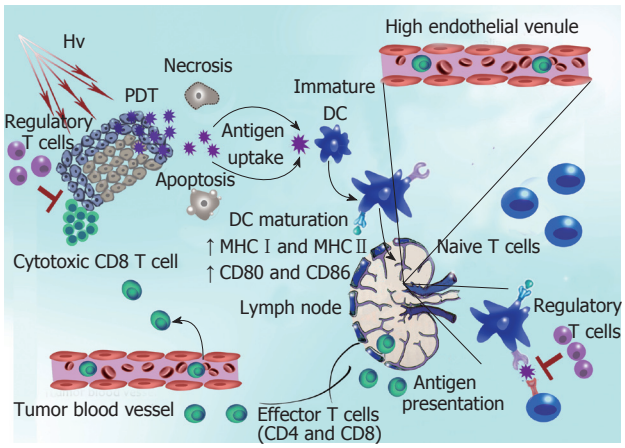

Figure 2 Stimulation of adaptive anti-tumor immunity by photodynamic therapy. PDT-treated tumor cells release the antigens, which are phagocytosed by DCs and presented to naive T cells in the tumor draining lymph node. Activated effector T cells return in circulation and migrate to the tumor. Regulatory T cells seem to inhibit the immune responses after PDT. DCs: Dendritic cells; Hv: Light; MHC Ⅰ: Major histocompatibility class Ⅰ; PDT: Photodynamic therapy. Original figure based upon Ref. [115] and Ref. [116].

massive infiltration of the immune cells into the tumor.

The inflammatory cells are known to be necessary to achieve efficacious PDT, as several studies have shown that their depletion (or inhibition of their activity) diminishes the therapeutic effect of the treatment[9,29,30]. Among all the cytokines involved in the PDT-induced inflammatory process, IL-1β and IL-6 seem to play the most important role[26,31] and conversely, IL-10 and transforming growth factor (TGF)-β seem to hamper PDT-effects as their blockade remarkably improves the cure rates after PDT[27]. Also, blocking the function of various adhesion molecules can affect the efficacy of PDT[26,32]. Figure 1 shows the important cells and mediators that are activated in the tumor environment after PDT of a tumor.

Although PDT is a local treatment, its effect is not limited to the local site, but it can induce a potent acute phase response with systemic consequences[33]. Studies in mouse models have shown that PDT leads to drastic rise in serum levels of acute phase reactants such as serum amyloid P components (SAP), C-reactive protein (CRP) and mannose-binding lectin A (MBL-A)[34]. SAP and CRP belong to the pentaxin family proteins and are involved in acute immunological responses[35]. They are specialized in facilitating the phagocytosis and removal of dying cells such as those killed in PDT-treated tumors. SAP production and release is a hallmark acute phase reactant response in mice, but in humans CRP is a more important acute phase reactant than SAP and PDT dose-dependent up-regulation of CRP has been demonstrated in human lung tumor A549 cells[35]. MBL-A is another important acute phase reactant with functional attributes similar to SAP[36].

Furthermore, a rapid increase in peripheral neutrophils is observed immediately after PDT and it is still present 24 h later, that is correlated with the influx of neutrophils into the treated tumors[37].

ADAPTIVE IMMUNE RESPONSES IN ANTI-CANCER PDT

The PDT-induced local and systemic inflammatory responses can enhance the development of an adaptive immune response capable of protecting the host organism in an antigen-specific manner, owing to immunological memory. It can be asked what is mediating the crosstalk between the innate and adaptive arms of the immune system after PDT. It has been realized that PDT enhancement of adaptive anti-tumor immunity involves the activation of DCs. DC are stimulated by the recognition of DAMPs/CDAMPs released and/or exposed by dying tumor cells[38]. One of the best characterized DAMPs induced by PDT is HSP70, which is released after PDT and forms stable chaperone complexes with cytoplasmic tumor antigens. Thereafter, the HSP-antigen complexes bind to the danger signal receptors, Toll-like receptors 2 and 4[39] on the surface of DCs, which are most potent antigen presenting cells (APCs). In the absence of inflammation DCs remain in an immature state, but when tissue inflammation and release of DAMPs occur, they mature and migrate in large numbers to the draining lymph nodes. The transition to the mature state of DC involves the upregulation of surface major histocompatibility class Ⅰ and Ⅱ molecules (MHC Ⅰ and MHC Ⅱ) and of the costimulatory molecules CD80 and CD86. These changes allow the DCs to express peptide-MHC complexes at the cell surface and prime efficiently $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) and hence to initiate an adaptive immune response. Figure 2 shows the process by which DCs engulf tumor antigens, become activated, traffic to lymph nodes where antigen specific T-cells proliferate and then return to attack remaining tumor cells.

The generation of $CD8^+$ effector and memory T cell

induction is generally, but not always dependent on $CD4^+$ helper T cells[40-42]. Kabingu *et al*[43] showed in fact that $CD8^+$ T cell-mediated immunity is independent of $CD4^+$ T cells and depends instead on natural killer cells. Also other studies suggest that $CD8^+$ cells play the most critical role in PDT mediated anti-tumor immunity, as in the absence of their activation and/or tumor infiltration the efficacy of PDT is reduced[9,43]. Furthermore, it has been shown that adoptive transfer of bare $CD8^+$ T cells to immunocompromised *scid* mice can significantly restore PDT efficacy[8].

The adaptive immunity is not provided only by antigen-specific T cells, but also by B cells. B cells produce antigen-specific immunoglobulins, mounting the so called humoral immune response. So far there is only one study showing that the activation of humoral immunity is implicated in the PDT-induced systemic antitumor protection, as seen by (1) increased serum IgG titers after PDT; (2) production of antibodies against existing antigens; and (3) marked B-cell infiltration in the tumor rim 24 h after PDT[44]. Nonetheless, the importance of the humoral components to the tumor eradication process remains unclear and needs further investigations.

ROLE OF TUMOR ANTIGENS IN THE ANTI-TUMOR IMMUNE RESPONSE

Tumor antigens (TAs) represent a sort of "bait" for the immune system, since they activate DCs and allow the antigen-specific CTLs to recognize and destroy the tumor cells. Some TAs have been well defined in murine and human tumors[45] and are generally classified in three distinct groups: (1) Antigens encoded by cancer-testis genes expressed in various tumors, but not in normal tissues, such as the mouse gene *P1A* and human genes of the melanoma antigen (MAGE)-type, B MAGE and G antigen families[46-51]; (2) Differentiation antigens of the melanocytic lineage, which are present on most melanomas but also on normal melanocytes (*i.e.*, melanoma antigen recognized by T-cells 1, gp100)[51-53]; and (3) Antigens that result from tumor-specific mutations in genes which are expressed in all tissues (*i.e.*, p53, p16) or come from viruses (*i.e.*, Epstein-Barr virus, Hepatitis B virus)[54-58]. Successful immunotherapeutic strategies targeting the TAs have been developed in preclinical studies and early-phase clinical trials[59,60], and our group was the first to realize the importance of TAs expression in PDT anti-tumor immunity.

We showed that a vascular PDT regimen was able to produce 100% of long term cures and rejection of re-challenge when tumors were induced in C3H mice with green fluorescent protein-expressing radiation-induced fibrosarcoma cells, but not with their wild-type counterpart[61]. The same effect was observed when we used a pair of equally lethal Balb/c colon adenocarcinomas: The antigen negative CT26 wild-type and the CT26.CL25 transduced with *lacZ* gene, and thus expressing the tumor antigen β-galactosidase[62]. We could further show that PDT of antigen positive tumors, but not of antigen negative tumors could trigger a highly potent antigen-specific systemic immune response capable to induce regression of distant untreated tumors. Recently we employed the P1A antigen positive mouse mastocytoma P815 wild-type and P1A antigen negative P1.204 (P815 derived) tumor models to study the antigen-specific PDT-induced antitumor immunity[63]. This model is clinically more relevant than others as the P1A is a naturally occurring murine cancer antigen, homologue of the human MAGE-type antigen[64]. We found that tumor cures, significantly higher survival and rejection of tumor rechallenge were obtained with P815, but not with P1.204 tumors that lack the antigen.

The role of the TAs in PDT anti-tumor immunity has been recently investigated also in the clinical setting. In a study published by Kabingu *et al*[65] in 2009, they demonstrated for the first time the enhancement of systemic immune reactivity to a basal cell carcinoma (BCC) associated TA (Hedgehog-interacting protein 1) following PDT in patients. These novel findings in patients are important as they are supporting the results in preclinical models, but more effort needs be done in clinical trials to elucidate the PDT-induced systemic immune responses to tumor antigen.

IMPACT OF T REGULATORY CELLS IN THE ANTI-TUMOR IMMUNE RESPONSE

In addition to directly stimulating anti-tumor immunity by triggering DCs and T cells activation, PDT may also interfere with immune-suppressive T cells. The main class of T cells suppressing the immune response consists of $CD4^+CD25^+FoxP3^+$ T regulatory cells (Treg)[66]. The involvement of Treg in both autoimmune disease[67] and cancer[68] has been extensively described in mice and humans. Treg are thought to mediate their immunosuppressive effects by multiple mechanisms[69]. Treg express the protein receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4), which is similar to the T-cell costimulator protein CD28. CTLA-4 binds with much higher affinity to B7-1 and B7-2 costimulatory molecules on APCs compared to the equivalent molecule CD28 and transmits inhibitory signals, rather than stimulatory[70].

Treg are generally classified into two main subpopulations: Natural Treg and induced Treg[71]; the former are found in the thymus and thought to have T-cell receptors that recognize self-antigens, therefore important in the prevention of autoimmune disease, the latter can be induced and differentiate in the periphery, *i.e.*, upon influence by TGF-β in the tumor microenvironment[72]. Several studies have shown that Treg inhibit the generation of immune responses against tumors[71], but on the other hand, their depletion *in vivo* facilitates tumor eradication and enhances-anti-tumor immunity[73-75]. A summary of the features of Treg is provided in Table 3.

Our research group was the first to investigate the potential relationship between PDT and Treg and we realized that Treg play an important and negative role in PDT anti-tumor immunity. We observed that if Treg are

Table 3 Common features of T regulatory cells

Features of Treg	Ref.
Phenotypic and functional specialization	[66-68]
Treg are $CD4^+CD25^+FoxP3^+$ immunosuppressive T cells. They are important for the maintenance of the immune homeostasis and involved in both autoimmune disease and cancer	[71,72]
Cells subpopulations	
Treg are generally classified into nTreg and iTreg. The former are found in the thymus and thought to have T-cell receptors that recognizes self-antigens, therefore important in the prevention of autoimmune disease, the latter can be induced and differentiate in the periphery, *i.e.*, upon influence by TGF-β in the tumor microenvironment	[69,70]
Immunosuppressive mechanisms	
Treg are thought to mediate their immunosuppressive effects by multiple mechanisms, among which	
Secretion of immunosuppressive cytokines	
High affinity binding of his CTLA-4 receptor to B7-1 and B7-2 costimulatory molecules on antigen presenting cells and transmission of inhibitory signals	
Role of Treg in anti-tumor immunity	[71,73-75]
Treg are known to inhibit the generation of immune responses against tumors. Treg depletion *in vivo* facilitates tumor eradication and enhances-anti-tumor immunity	

nTreg: Natural Treg; iTreg: Induced Treg; CTLA-4: Cytotoxic T-lymphocyte antigen 4; Treg: T regulatory cells; TGF: Transforming growth factor.

depleted by low-dose cyclophosphamide (CY) (a traditional cytotoxic cancer drug that at low doses selectively depletes Treg[76]) prior to PDT, the anti-tumor immune responses are potentiated and a memory immunity is generated against metastatic J774 tumors[77]. This effect was not seen when PDT and CY treatments were given separately or when PDT was combined with high-dose CY that destroyed all T-cells not just Treg. Another recently completed study involving the colon adenocarcinoma CT26 wild-type tumor model revealed that the combination of PDT with low-dose CY produced a dramatic improvement in long-term survival, compared with either treatment alone and led the development of immune response to the mouse cancer shared/auto-antigen gp70[78]. Moreover, this combination treatment activated a long-lasting immune memory, that could however be uncovered only when Treg were depleted again by CY before rechallenge. These new findings are important, because they emphasizes that one of the most effective approaches for optimally improving anti-cancer PDT would be by restraining host's regulatory immune cell populations.

CLINICAL EVIDENCE FOR THE IMPACT OF THE IMMUNE SYSTEM IN ANTI-CANCER PDT EFFECTS

The first clinical use of PDT for cancer in modern times dates back to the beginning of the 20th century when Von Tappeiner *et al*[79] used eosin as topical PS combined to sunlight to treat facial BCC. That first trial was successful as out of 6 patients, 4 showed complete tumor resolution. Many years later, in the 1970s, hematoporphyrin derivative (HPD) and light were administered to the tumor area of patients with bladder cancer[80] and resulted in positive outcomes. In the same decade Dougherty *et al*[81] tested for the first time HPD-PDT in a large series of patients with skin tumors reporting striking results: Complete or partial responses were observed in 111 out of 113 patients.

Since then, over 200 clinical trials for PDT as treatment for a large variety of tumors have been carried out. Some clinical studies have demonstrated that PDT efficacy seems to depend on antitumor immunity also in patients. Dragieva *et al*[82] published a study comparing the efficacy of PDT for actinic keratosis and Bowen's disease in immune-competent patients *vs* immune-suppressed transplant patients. The two groups of patients showed comparable initial response, however the immune-suppressed patients had an increased propensity to develop new lesions after the treatment. It has also been shown that patients with vulval intraepithelial neoplasia (VIN) expressing MHC I molecules on the tumor cells were more likely to respond to aminolevulinic acid-PDT compared to patients whose tumors had down-regulated MHC I molecules[83]. MHC I recognition is critical for activation of $CD8^+$ T cells and the down-regulation of MHC I molecules is one of the mecha-nisms used by tumors to evade immune recognition in general and PDT-induced immunity in particular. VIN patients who did not respond to PDT had significantly lower $CD8^+$ T cell infiltration into the treated tumors compared with responders, confirming the important role of $CD8^+$ CTLs in PDT efficacy. The first clinical case of systemic PDT-immune response observed in patients has been published in 2007: PDT of multifocal angiosarcoma of the head and neck located on the right upper limb of a patient, resulted in a spontaneous regression of the untreated distant tumors on the contralateral left upper limb, accompanied by increased immune cell infiltration[84]. Two years later Kabingu *et al*[65] found that PDT treatment of BCC lesions enhanced the reactivity of patients lymphocytes against Hip1, a known BCC-associated TA, as seen by increased secretion of IFN-γ by patients lymphocytes following incubation with the TA derived peptide.

PDT FOR INFECTIONS

Although PDT was discovered in the field of microbiology over 100 years ago[85], up to now PDT has been

studied and applied mainly as anticancer treatment. The discovery of antibiotics in 1940s revolutionized the treatment of infectious disease, limiting the development of other potential alternative anti-microbial treatments like PDT. However, the recent worldwide increase of resistance to antibiotics has strongly enhanced the interest in alternative therapeutic strategies for the treatment of infections. PDT is capable of killing a large variety of pathogens such as bacteria, parasitic protozoa, fungi, yeasts and viruses. Furthermore, PDT does not induce resistance itself and it is a non-invasive method. PDT is more effective in inactivating Gram (+) bacteria compared to Gram (-) due to the different structure of the cell walls[86]. The membrane of Gram (+) bacteria is surrounded by a permeable layer of peptidoglycan and lipoteichoic acid that allows the PS to pass through it[87]. Gram (-) species have an inner cytoplasmic membrane and an outer membrane, which are separated by a peptidoglycan-containing periplasm. The outer membrane constitutes a permeability barrier between the cell and its environment, limiting the PS penetration. Fungal cell walls have a moderately thick layer of chitin and β-glucan that result in a barrier with moderate permeability. Several *in vitro* and *in vivo* studies have been carried out to verify the efficacy of PDT for viral infections, soft tissues infections, oral and dental infections produced by different strains of bacteria. PDT has been shown to work efficiently against *Escherichia coli* and *Pseudomonas aeruginosa* in excisional wounds[88,89] and against *Acinetobacter baumannii* and *Staphylococcus aureus* in burn infections[90,91].

There are reports of PDT on its effects on certain species of fungus, including both filamentous fungi (*Trichophyton*[92] and *Aspergillus*[93]) and yeasts (*Saccharomyces*[94] and *Candida albicans*[95,96]). Also several types of virus have been tested for the affection by PDT, including herpes viruses HSV-1[97] (PDT by methylene blue and light), enveloped RNA viruses from two different families, Semliki Forest Virus (*Togaviridae*) and vesicular stomatitis virus (*Rhabdoviridae*) (PDT by buckminsterfullerene and light)[98] and others[99].

Some clinical trials for PDT have been carried out for dental, gastric and dermatological infections such as acne as well as rosacea, a condition in which microbes may play a role in the pathogenesis[100].

IMMUNE RESPONSES IN ANTI-BACTERIAL PDT

While the immune stimulating effects of PDT have been widely studied in cancer models, little is known about the immunological effects of PDT in bacterial infections. A recent study published by Tanaka *et al*[10] convincingly demonstrated for the first time that *in vivo* PDT can stimulate an innate immune response. They used a mouse model of bacterial arthritis (*Staphylococcus aureus* infection in the knee joint) and observed a strong infiltration of neutrophils in the PDT-treated area. In order to investigate the role of neutrophils in the PDT-mediated bacteria inactivation, they administered anti-GR-1 (anti-neutrophil) antibody as well as antibodies to several pro-inflammatory mediators. The administration of such antibodies resulted in loss of the therapeutic effect of PDT. This suggests that not only killing of bacteria, but also attraction and accumulation of neutrophils into the infected regions were required mechanisms to achieve PDT-mediated clearance of bacterial infections. Additionally, PDT was tested also as a preventive therapeutic approach and delivered prior to the bacterial inoculation into the knee. PDT-mediated infiltration of neutrophils prevented the subsequent inoculation of bacteria from establishing the infection and again, such an effect was abrogated when antibodies against GR-1 and proinflammatory mediators were administered. To the best of our knowledge, this is the first demonstration of a protective innate immune response against a microbial pathogen being induced by PDT. It is well known that bacterial phagocytosis by innate immune cells such as neutrophils, plays a crucial role in the elimination of invading bacteria and, therefore, malfunction of the phagocytic immune system renders the host more susceptible to bacterial infections. Hence, it would be desirable to apply an antimicrobial PDT regimen that causes direct photoinactivation of bacteria, but at the same time that can minimize the damage to the host's neutrophils.

As described above, evidence indicated that PDT of cancer triggers the activation of both innate and adaptive arms of the immune system, while the early results from the bacterial infection models suggest that PDT is capable of stimulating (at least) the innate immune system. The biggest difference, however, could be in the stimulation of T- and B-cell-mediated adaptive immune responses. As antibodies produced by B cells are generally the most effective component of the immune response against bacterial infection, B cells are expected to be the main actors in the post-PDT immune response towards bacteria. However, to the best of our knowledge, nothing is known yet about humoral responses induced by PDT against bacterial infection.

On the other side, while the involvement of B cells in PDT-induced antitumor immunity still needs more investigation, it is widely accepted that the activation of T cell responses play a pivotal role in PDT-mediated immunity towards treated tumors.

CONCLUSION

Several studies in pre-clinical and clinical settings have demonstrated that PDT is capable of pronouncedly activating both the innate and adaptive arms of the immune system. Such effects on the immune system appear to be PDT regimen dependent and strictly linked to the degree of inflammation induced by PDT.

It has been speculated that PDT regimens causing a high degree of acute inflammation are better at immune activation compared to those in which the acute inflammation is lower. However, increase in inflammatory

mediators could promote tumor cell growth in certain circumstances[101]. Moreover, PDT has been linked also to immunosuppressive effects. Such immunosuppressive effects have been established in model of suppression of induction of contact hypersensitivity (*i.e.*, afferent immune response), which involves the application of a hapten to the skin, followed by re-challenge[102], and suppression of delayed-type hypersensitivity (Mantoux) reactions (*i.e.*, efferent immune response) for instance in healthy Mantoux-positive volunteers[103,104]. In particular, such immunosuppressive responses seem to be dependent on the rate of light delivery[105] and anatomic site of PDT[106].

Further studies using a better targeted and dose-controlled PDT treatment would help to expand the knowledge on the activation/suppression of the immune system and the possibilities to improve it in clinical practice.

The proven ability of PDT to trigger inflammation and improve the anti-tumor immune response could be successfully employed in tandem with other treatment modalities, to combat cancer and to achieve long-term tumor control. Nevertheless, up to now PDT remains clinically underutilized. We must realize that with all probability it will take several years of further investigations and clinical trials before the use of PDT becomes a clinically accepted standard practice in cancer patients.

The innate immune responses seem to be of crucial importance also in the relatively new field of PDT as anti-microbial treatment. The activation of neutrophils after PDT, their mobilization from the bone marrow and their attraction to the site of inflammation appear to be important mechanisms, significantly potentiating the antibacterial effects, *e.g.*, in bacterial arthritis mouse models. However, it still remains to be elucidated whether the activation of the host neutrophils is applicable also to other infection models, with other classes of pathogens and/or using different PS. Many years of intense research will be required providing answers to these intriguing questions.

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P- Reviewers: Kwon HJ, Nagata T, Yun CH **S- Editor**: Song XX
L- Editor: A **E- Editor**: Liu SQ

World Journal of Immunology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5411/wji.v4.i1.12

World J Immunol 2014 March 27; 4(1): 12-19
ISSN 2219-2824 (online)

REVIEW

Circulating immune cell activation and diet: A review on human trials

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Received: October 21, 2013 Revised: December 5, 2013
Accepted: February 16, 2014
Published online: March 27, 2014

Abstract

Protein energy malnutrition is the main cause of immunodeficiency and, secondarily, of several infections. However, immune cell activation is involved in several pathophysiological processes that play a crucial role in the appearance of cardiovascular disease (CVD) or cancer. The aim of this review is to update the knowledge of the modulation of immune cell activation by different dietary patterns and its components focusing on CVD or cancer. While a westernized high-saturated fat high-carbohydrate diet is positively associated with low-grade inflammation, vegetable- and fruit-based diets rich in monounsaturated fatty acids, polyunsaturated fatty acids and polyphenols, key nutrients of Mediterranean diet, decrease the levels of cellular and circulating inflammatory biomarkers thereby reducing the risk of related chronic diseases.

Key words: Mediterranean diet; Immune cell activation; Leukocyte adhesion; Inflammation; Fatty acids; Fruits; Vegetables; Polyphenols; Cardiovascular disease; Cancer

Core tip: Immune cell activation is involved in several pathophysiological processes that play a crucial role in the appearance of cardiovascular disease or cancer. The aim of this review is to update the knowledge of the modulation of immune cell activation by different dietary patterns. A westernized high-saturated fat high-carbohydrate diet is positively associated with low-grade inflammation, but a Mediterranean diet, rich in vegetables and fruits decrease the levels of cellular and circulating inflammatory biomarkers thereby reducing the risk of related chronic diseases.

Chiva-Blanch G, Estruch R. Circulating immune cell activation and diet: A review on human trials. *World J Immunol* 2014; 4(1): 12-19 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i1/12.htm DOI: http://dx.doi.org/10.5411/wji.v4.i1.12

INTRODUCTION

Although some food compounds can originate several immune reactions such as allergies or celiac disease[1], most of nutrients in the diet are essential for maintaining the function of immune cells. Thus, protein energy malnutrition is the main cause of immunodeficiency worldwide[2] and deficits of nutrients commonly included in the diet such as vitamin E, vitamin C, β-carotene, selenium, copper, iron and zinc modify different immune functions related to infections caused by bacteria, viruses or parasites. A deficiency in vitamin E diminishes the ability of the immune system to respond to infectious microorganisms and under some conditions, a pharmacological level of vitamin E is needed to achieve an optimal immune response suggesting that the recommended dietary allowances for vitamin E might not be adequate for immuno-

logical vigor and health[3]. Therefore, the knowledge of the effects of nutrition on immune function now extends beyond clinical nutrient deficiency. A growing body of literature demonstrates the immune benefits of increasing the intake of some specific nutrients. Compared with human infants fed formula without nucleotides, infants fed breast milk or formula supplemented with nucleotides had higher natural killer cell activity and interleukin (IL)-2 production[4] and consequently, improved immune function. On the other hand, in an elderly population, zinc supplementation eliminated the effect of seasonal variations on the incidence of infections and also decreased their mean incidence compared to a placebo group (common cold, cold sores, and the flu)[5].

In addition to protection against infections, immune cell activation is involved in several pathophysiological processes. Cell activation is a complex process, implying several plasma membrane-associated events in which chemokines and adhesion molecules play a pivotal role. These processes ultimately result in proliferation, target cell lysis, increased production of cytokines and the expression of immune cell activation markers.

Many chronic diseases, such as atherosclerosis, cancer, neurodegenerative disorders, rheumatoid arthritis, and even aging, are due to chronically increased pro-inflammatory cytokines and oxidative stress, and in consequence, due to immune cell activation. Therefore, dietetic strategies to decrease low-grade inflammation and immune cell activation may be useful tools for preventing or decreasing the progression of many chronic disorders.

Many studies have focused on the mechanisms by which one single nutrient or compound alters immune cell activation, but these studies have the limitation that the interactions between the different compounds of food are not considered. There is an increasing interest to consider a whole dietary pattern in addition to single compounds in order to have a holistic approach of the effects of diet on cell activation.

The aim of this review is to update the knowledge of the modulation of immune cell activation by diet and its components from a chronic disease point of view through human interventional studies, those which provide the greatest scientific evidence.

EFFECTS OF DIET ON INFLAMMATORY CHRONIC DISEASES

Chronic inflammatory diseases are defined by long-term inflammatory processes directed at a particular endogenous or exogenous antigen and considered as an underlying pathophysiological mechanism linking behavioral factors and obesity to risk of chronic disease. Inflammation is characterized by a complex biological cascade of molecular and cellular signals that alter physiological responses. At the site of the injury, cells release molecular signals such as cytokines that cause a number of changes in the affected area, such as dilation of blood vessels, increased blood flow, increased vascular permeability, exudation of fluids containing antibodies and invasion by monocytes and macrophages and, to a lesser extent, lymphocytes, through the expression of integrins and other adhesion molecules. In addition, lesion progression is associated with the predominance of the proinflammatory M1 over the antiinflammatory M2 macrophage phenotype, which can be switched to M1 by several transcription factors, chemokines and lipid accumulation in macrophages[6,7].

Elevated levels of inflammatory biomarkers such as C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), IL-6 and 18, fibrinogen and adhesion molecules [E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1)] have been shown to predict type 2 diabetes, cardiovascular disease (CVD) and cancer[8-13]. In this setting, the activation of the inflammasome has been linked to the pathogenesis of obesity, type 2 diabetes and atherosclerosis[14-19]. Several studies have suggested that the development of tolerance and control of inflammation are strongly correlated with specific immune mechanisms that may be altered by an inadequate supply of either macronutrients or micronutrients. Therefore, the intake of some nutrients or specific dietary patterns may influence the concentrations of inflammatory biomarkers and therefore, the risk and/or progression of inflammatory diseases.

A westernized high-fat high-carbohydrate diet is positively associated with low-grade inflammation, and therefore, contributes to disease development and progression. Likewise, these types of diets can have direct adverse effects on human physiology[20] resulting in chronic immune and inflammatory imbalances. Overall, the intake of a high-fat, high-carbohydrate westernized diet has potent direct and indirect effects on local as well as systemic inflammation. This has led to a dramatic upswing in the incidence of inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, diabetes, and non-alcoholic fatty liver disease, among others[21]. On the other hand, vegetable- and fruit-based diet and a priori healthy dietary patterns appeared to be inversely related to inflammatory biomarkers; this fact is particularly well supported by intervention studies investigating the effects of Mediterranean diet (MedDiet) on health.

EFFECTS OF DIET ON CVD AND METABOLIC SYNDROME

CVD is the main cause of mortality worldwide and is principally caused by the appearance and progression of atherosclerotic lesions. Although atherosclerosis has been historically considered an oxidative disease, nowadays it is considered a systemic disease characterized by low-grade arterial inflammation, in which the cell and endothelial expression of adhesion molecules and chemokines participate in the recruitment of circulating leukocytes to the vascular endothelium and further migration into subendothelial spaces. In addition, the metabolic syndrome is

a risk factor CVD, which also has an immunological and inflammatory component. Immune cell infiltration of adipose tissue giving rise to chronic low-grade inflammation is, in part, responsible for the pathogenesis of insulin resistance in obesity[22] and lastly, CVD.

As explained before, the western diet enriched in total fat (and an imbalanced ratio of n-6:n-3[23]), animal protein, n-6 polyunsaturated fatty acids (PUFA) and refined sugars, leads to an increased proinflammatory status[24] and is, therefore, considered as a risk factor for the development of CVD[25]. On the other hand, several studies have highlighted that a Mediterranean-like diet decreases cardiovascular risk[26-29], by up to 30% in a high cardiovascular risk population[30]. Several mechanisms have been proposed for the effects observed, and some of them, which will be discussed below, are related to immune cell activation.

While n-6 PUFA have been shown to exert an inflammatory effect[31], it has been demonstrated that n-3 PUFAs have beneficial effects on cardiovascular and inflammatory diseases[32,33], probably linked to the nuclear factor (NF)-κB pathway[34] and the inhibition of the inflammasome activation[35]. Meta-analyses of randomized controlled trials studying the n-6 PUFA-specific effect on CVDs[36] showed a direct effect of n-6 fatty acids on the risk of non fatal and fatal heart failure, although linoleic acid could not be linked to an increase in systemic inflammatory biomarkers[37]. Hypercholesterolemic subjects receiveing 2 diets low in saturated fat and cholesterol, and high in PUFA varying in α-linolenic acid (10.5% linoleic acid; 6.5% α-linolenic acid) and linoleic acid (12.6% linoleic acid; 3.6% α-linolenic acid) were compared with other who followed an average American diet (7.7% linoleic acid; 0.8% α-linolenic acid). The α-linolenic acid diet decreased circulating CRP, VCAM-1 and E-selectin plasma concentrations, and the 2 high-PUFA diets similarly decreased ICAM-1[38], although other studies in hypercholesterolemic subjects observed no such results[39]. In healthy women, a 2-wk intervention of a n-3 PUFA-enriched juice or a plain tomato juice decreased VCAM-1 levels but only the n-3 PUFA-enriched juice decreased ICAM-1 plasma concentrations[40]. In addition, a high monounsaturated fatty acid (MUFA) intake has also been shown to exert anti-inflammatory effects. A crossover feeding trial observed that a breakfast rich in butter [saturated fatty acids (SFA)] increased leukocyte mRNA expression of TNF-α compared to an olive oil (rich in MUFA, and concretely oleic acid) or walnut breakfast (rich in PUFA n-6)[41]. On the other hand, in overweight men, a low-fat and a very-low-carbohydrate diet resulted in significant decreases of TNF-α, IL-6, CRP and sICAM-1 but not P-Selectin[42], although in another study with overweight or obese women with metabolic syndrome, the substitution of carbohydrates by PUFA resulted in no changes in CRP, TNF-α, IL-6, sICAM-1 and sVCAM-1 serum concentrations[43]. It can be summarized that the overall quantity of fat intake, the sources and type of dietary fat, with special emphasis on α-linolenic acid and oleic acid, and the ratio of n-6:n-3 fatty acids in the diet, collectively play a crucial role in modulating inflammation.

Other dietetic compounds influencing immune cell activation are polyphenols. These products are antioxidant phytochemicals that have been found in vegetables, fruits and derivatives such as cocoa, red wine or tea, shown to decrease TNF-α and CRP levels[44]. In healthy volunteers cocoa consumption reduced NF-κB activation in peripheral blood mononuclear cells[45], and in men at high CVD risk, cocoa consumption decreased monocyte expression of very late antigen (VLA)-4, CD40 and CD36 and serum concentrations of P-selectin and ICAM-1[46]. Grape polyphenols and specially resveratrol are among the polyphenols most frequently studied. In hemodialysis patients, red grape juice supplementation for 3-wk significantly reduced plasma monocyte chemoattractant protein 1 (MCP-1)[47], and in overweight or obese subjects with metabolic syndrome, grapefruit supplementation for 6-wk decreased F2-isoprostane concentrations in those subjects with high baseline F2-isoprostane concentrations, but no changes in CRP and VCAM-1 were observed[48]. These results suggest different responses to polyphenol intake depending on the pathophysiological conditions of the study subjects and probably the type of polyphenols administered in the intervention group. These differential effects were also observed after moderate red wine consumption, where in healthy male volunteers red wine consumption significantly reduced plasma concentrations of VCAM-1, ICAM-1 and IL-1α and VLA-4 lymphocyte expression and lymphocyte function-associated antigen (LFA)-1, Mac-1, VLA-4 and MCP-1 monocyte expression[49]. On the other hand, in high cardiovascular risk subjects, moderate red wine consumption and dealcoholized red wine consumption (therefore, the non alcoholic fraction of red wine, mainly polyphenols) decreased serum concentrations of CD40 antigen, CD40 Ligand, ICAM-1, E-Selectin, IL-16 and IL-6, MCP-1 and VCAM-1 and inhibited the expression of LFA-1 in T-lymphocytes and Mac-1, SLe^x and C-C chemokine receptor type 2 expression in monocytes[50].

In the recent years, the effects of a MedDiet as a dietary pattern and not a sum of nutrients have been considered from a multidisciplinary point of view. The MedDiet is characterized by a high intake of cereals, fruit and vegetable products (and therefore, polyphenols), a moderate consumption of fish, olive oil, nuts and wine, and a low intake of meat and dairy and industrial bakery products[51]. According to scientific evidence, the MedDiet is currently considered the more anti-inflammatory dietary pattern, and this is translated to a decreased risk in cardiovascular mortality[30]. In patients with metabolic syndrome, a 2-year follow-up MedDiet reduced serum concentrations of CRP, IL-6, IL-7 and IL-18, accompanied with decreased insulin resistance and an improved endothelial function score[52]. In older subjects with diabetes or ⩾ 3 CVD risk factors randomly allocated to a 3-mo MedDiet with supplemented with extra-virgin

olive oil, a MedDiet supplemented with nuts or a low-fat diet, after both MedDiets CRP, IL-6, ICAM-1 and VCAM-1 plasma concentrations decreased as did CD40 and CD49d monocyte expression, whereas IL-6, ICAM-1 and VCAM-1 increased after the low-fat diet[26]. In addition, after 1 year both MedDiet groups showed lower plasma concentrations of IL-6, tumor necrosis factor receptor 60 (TNFR60), and TNFR80, whereas ICAM-1, TNFR60, and TNFR80 concentrations increased in the low-fat diet group[27]. The MedDiet has also shown anti-inflammatory effects in healthy subjects. Four weeks of a MedDiet compared to an ordinary Swedish diet decreased the number of platelets and leukocytes and serum concentrations of vascular endothelial growth factor (VEGF), although it did not change the CRP and IL-6 concentrations[28], perhaps because of their low baseline concentration. Interestingly, in a middle-aged twin population, adherence to a MedDiet was highly associated with lower levels of IL-6 but not CRP[29]. Overall, the MedDiet has an antiinflammatory and an inhibitory immune cell activation effect decreasing the onset and progression of CVD, while a low-fat diet or a westernized diet has the opposite effect.

EFFECTS OF DIET ON CANCER

Cancer is the second cause of mortality worldwide and is mediated by both the innate (nonspecific) and acquired (specific) immune systems[53,54]. The molecular mechanisms by which chronic inflammation drives cancer initiation and promotion include increased production of pro-inflammatory mediators, such as cytokines, chemokines, reactive oxygen intermediates, increased expression of oncogenes, cyclooxygenases, lipoxygenases and matrix metalloproteinases, and pro-inflammatory transcription factors such as NF-κB, that mediate tumor cell proliferation, transformation, metastasis, survival, invasion, angiogenesis, chemoresistance and radioresistance[55]. Taking into account that adherence to ideal cardiovascular health, as proposed by the American Heart Association, is associated with a lower incidence of cancer[56], one may suspect that dietary benefits on CVD may reduce the risk of cancer.

Although epidemiological studies have pinpointed that diet may influence more than one-third of human malignancies, probably through the high consumption of pesticides[57], heavy metals[58], heterocyclic amines from over-cooked meats and sex steroid hormones[59], few interventional trials have focused on the modulation of angiogenesis and carcinogenesis through dietary patterns. A recent review[60] of epidemiological studies concluded that there is no significant effect of n-3 PUFA on cancer risk. However these studies only accounted for absolute as opposed to relative levels of n-3 and n-6 PUFA. In fact, n-6 PUFA metabolites promote tumor angiogenesis through a variety of signaling pathways, encouraging epithelial cell proliferation and migration, and decreasing tumor apoptosis, while n-3 PUFA and their metabolites can reverse the pro-angiogenic consequences of high n-6 fatty acids. On the other hand, a MedDiet supplemented with nuts and walnuts (rich in n-6 and polyphenols) also associated with a high intake of vegetables, fruit and fish, decreased the risk of cancer mortality[61].

Intake of total catechin, epicatechin, kaempferol, and myricetin and consumption of black tea were associated with a decreased risk of stage Ⅲ/Ⅳ or stage Ⅳ prostate cancer in the Netherlands Cohort study[62], probably because of the anti-inflammatory and antiproliferative effects of flavonoids observed *in vitro*[63]. In prostate cancer men, 30 d of low-fat diet decreased 19 cytokines and angiogenic factors including proangiogenic factors (stromal-cell derived-1α) and myeloid factors [granulocyte-colony-stimulating factor, macrophage colony-stimulating factor (-M-CSF-)] and VEGF, probably through the NF-κB pathway[64]. Regarding breast cancer, diets high in n-6 PUFA have a clear stimulating influence on breast cancer development, whereas diets rich in extra virgin olive oil mainly have a negative modulatory effect[65]. A recent meta-analysis[66], showed a significant inverse association with the highest fiber intakes and the risk of esophageal cancer, probably through weight control and therefore, inflammation status control. The isothiocyanate sulforaphane [SF; 1-isothiocyanato-4(R)-methylsulfinylbutane] is abundant in broccoli sprouts in the form of its glucosinolate precursor (glucoraphanin). SF is powerful bactericidal against *Helicobacter pylori* (*H. pylori*) infections, which are strongly associated with the worldwide pandemic of gastric cancer. *H. pylori*-infected patients were randomly assigned to feeding of broccoli sprouts (70 g/d; containing 420 micromol of SF precursor) for 8 wk or to consumption of an equal weight of alfalfa sprouts (not containing SF) as placebo. Intervention with broccoli sprouts, but not with alfalfa, decreased the levels of urease measured by the urea breath test and *H. pylori* stool antigen (both biomarkers of *H. pylori* colonization) and serum pepsinogens I and Ⅱ (biomarkers of gastric inflammation). Therefore, daily intake of SF-rich broccoli sprouts for 2 mo enhanced the chemoprotection of the gastric mucosa against *H. pylori*[67]. In colorectal cancer (CRC) patients, drinking a slurry of black raspberry powder 3 times-a-day for 9 wk increased granulocyte -M-CSF- and decreased IL-8 plasma concentrations and CD105 colorectal tissue expression[68], while in another large CRC cohort, red and processed meat intake before CRC diagnosis was associated with higher risks of death due to all causes and from CVD but not CRC. Although red and processed meat consumption after CRC diagnosis was not associated with mortality, survivors with consistently high (median or higher) intakes before and after diagnosis had a higher risk of CRC-specific mortality compared with those with consistently low intakes[69]. Nonetheless, it should be taken into account that, in addition to the diet, colon cancer risk is influenced by the balance between microbial production of health-promoting metabolites and potentially carcinogenic metabolites[70]. In summary, few interventional studies have been per-

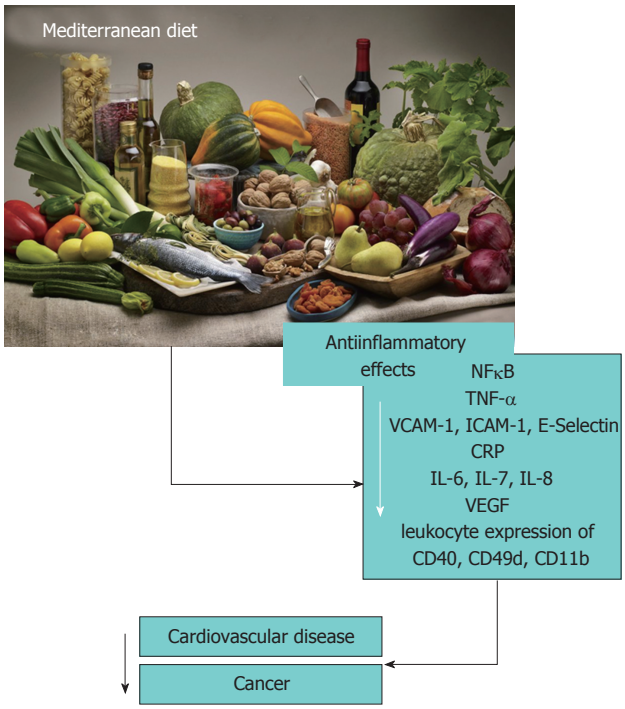

Figure 1 Summary of the anti-inflammatory effects of the Mediterranean Diet. CRP: C-reactive protein; TNF-α: tumor necrosis factor-α; ICAM-1: Intercellular adhesion molecule 1; VCAM-1: Vascular cell adhesion protein 1; IL-6: Interleukin 6; VEGF: Vascular endothelial growth factor; NF: Nuclear factor.

formed investigating the link between diet, immune cell activation and cancer, but it can be postulated that a MedDiet brings together all the dietary protective nutrients related to cancer and specially cancers of the digestive system decreasing its risk of appearance[71], although there are still not enough data to develop guidelines regarding specific foods and cancer risk.

FUTURE PERSPECTIVES

Dietary intake in relation to low-grade inflammation has been investigated in a number of studies exploring nutrients, foods or dietary patterns. Although there is increasing evidence that dietary patterns modulate immune cell activation and low-grade systemic inflammation, there is still a long way to understand the interactions between dietary compounds, dietary patterns, microbiota metabolites and individual polymorphisms and how these affect the body response to the intake of a determined food compound. The integration of dietary behaviors is warranted, given the fact that nutrients or foods are rarely eaten alone, and dietary patterns consider synergistic or antagonistic biochemical interactions among nutrients as well as different food sources of the same nutrient.

CONCLUSION

As summarized in Figure 1, there is compelling scientific evidence that a MedDiet rich in MUFA, PUFA (with an adequate ratio of n-3:n-6), polyphenols and with mild-to-low carbohydrate, animal protein and SFA content is the most effective pattern to prevent immune cell activation and inflammation related to chronic diseases by decreasing the expression of leukocyte adhesion molecules and circulating inflammatory biomarkers.

ACKNOWLEDGMENTS

CIBERobn is an initiative of Instituto de Salud Carlos Ⅲ, Spain.

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P- Reviewers: Kucuker A, Kindy MS, Movahed A
S- Editor: Song XX **L- Editor**: A **E- Editor**: Liu SQ

World Journal of Immunology

Online Submissions: http://www.wjgnet.com/esps/
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doi:10.5411/wji.v4.i1.20

World J Immunol 2014 March 27; 4(1): 20-25
ISSN 2219-2824 (online)

MINIREVIEWS

Cigarette smoking and innate immune responses to influenza infection

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Supported by The Oklahoma Health Research Program from Oklahoma Center for the Advancement of Science and Technology, to Wu W; and by the National Institute of Allergy and Infectious Diseases, No. 1U19 AI62629, to Metcalf JP
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Received: October 31, 2013 Revised: December 18, 2013
Accepted: February 16, 2014
Published online: March 27, 2014

Abstract

Cigarette smoking (CS) suppresses the immune system, and smoking is a well-known major risk factor for respiratory tract infections, including influenza infection. Both smoking cigarettes and passive smoking alter a wide range of immunological functions, including innate and adaptive immune responses. Past reviews on CS and innate immunity have been focused on the effects of CS on structural changes of the lung, as well as the effects on the function of alveolar macrophages, leukocytes, natural killer cells and dendritic cells. The study of innate immunity has developed rapidly in the last decade with the discovery of new receptors for virus recognition and interferon responses. This review aims to give a brief summary of recent findings on the suppressive effects of CS on the innate response to influenza virus, especially as it pertains to suppression of the function of pattern recognition receptors for influenza virus.

Key words: Smoking; Influenza; Innate immunity; Virus; Pattern recognition receptors; Immune response

Core tip: Cigarette smoking (CS) alters a wide range of immunological functions, including innate and adaptive immune responses to viral infection. This review aims to give a brief summary of recent findings on the suppressive effects of CS on the innate response to influenza virus, especially as it pertains to suppression of the function of pattern recognition receptors for influenza virus. Studies on CS inhibition to innate response will be important in designing strategies for the development of novel treatments to mitigate the adverse consequences of CS and Flu infection.

Wu W, Metcalf JP. Cigarette smoking and innate immune responses to influenza infection. *World J Immunol* 2014; 4(1): 20-25 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i1/20.htm DOI: http://dx.doi.org/10.5411/wji.v4.i1.20

CIGARETTE SMOKING AND INFLUENZA INFECTION

Influenza virus is a major cause of infectious morbidity and mortality[1]. Each year in the United States, 5% to 20% of the population are infected, 200000 are hospitalized, and 36000 die due to influenza virus infection, making it the leading infectious cause of death[2,3]. There have been four pandemics (worldwide epidemics) in the last century, including the Spanish flu in 1918, the Asian flu in 1957, the Hong Kong flu in 1968, and the Swine flu in 2009. These were significant outbreaks. For example,

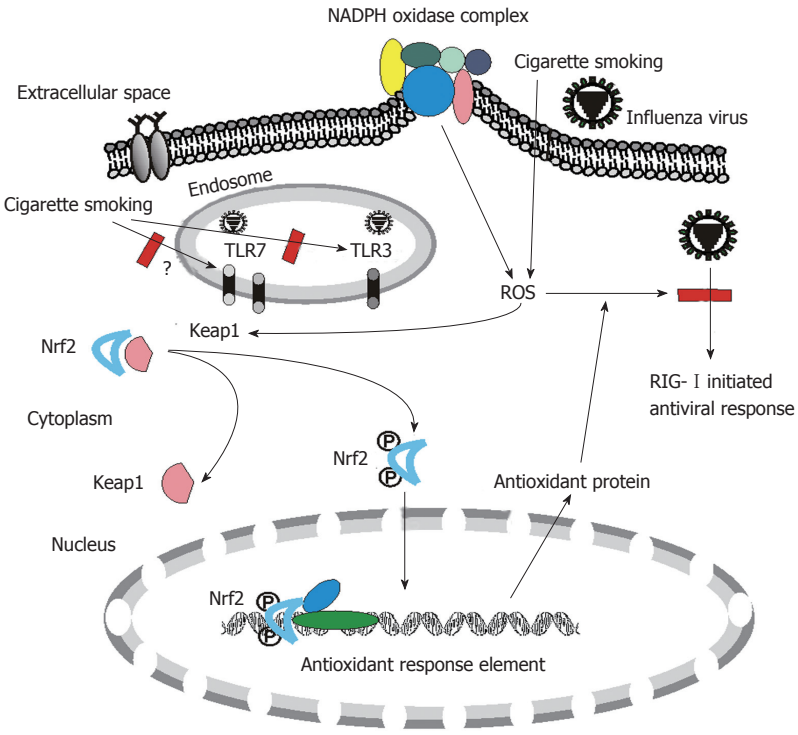

Figure 1 Cigarette smoking and its subsequent induced cellular oxidative stress suppress innate response to influenza virus. Influenza virus components are internalized from the cell surface in endosomes, and specific ligands are recognized by toll-like receptors (TLR)3/7 and cytosolic retinoic acid-inducible gene 1 (RIG- I). The PRRs then activate transcription factors that lead to the production of antiviral interferons and pro-inflammatory cytokines. Cigarette smoking (CS) inhibits RIG- I , TLR3 and possibly TLR7 recognition of influenza virus. Reactive oxygen species induced by CS are involved in the interference of PRR function. Reducing oxidative stress in cells, either by increasing Nrf2 or by Keap1 knockout, has potential therapeutic effect of restoring virus recognition by PRRs suppressed by CS. ROS: Reactive oxygen species; PRRs: Pattern recognition receptors.

the 1918 flu caused more deaths than those due to World War I . Influenza pandemics will continue as a threat to public health. The predisposition of cigarette smokers to have, and to have complications from, influenza infection is well recognized[4]. Epidemiological studies show that influenza infection is seven times more common and is much more severe in smokers than nonsmokers[5]. Influenza infections are more severe, with more cough, acute and chronic sputum production, breathlessness, and wheezing in smokers[6]. Both active and passive cigarette smoke exposure increase the risk of infections[7]. A cohort study of female military recruits showed that smoking was a risk factor for severe influenza-like illness during an outbreak of influenza A (H1N1) subtype infection[8]. Thailand's National Avian Influenza Surveillance system reported that current or former smoking was among the several risk factors associated with a fatal outcome from human influenza infection[9]. In the spring of 2013, the high mortality of avian influenza H7N9 in humans caused great concern in China and the world. Age along with a history of smoking are the most significant risk factors which predict a fatal outcome in human H7N9 infection[10]. The mechanism of increased susceptibility to influenza infections in smokers is likely multifactorial, but clearly includes alteration of immunologic host defenses. Both smoking cigarettes and second-hand exposure to tobacco smoke alter a wide range of immunological functions, including innate and adaptive immune responses[11].

CIGARETTE SMOKING SUPPRESSES INNATE RESPONSE RECEPTORS TO INFLUENZA VIRUS

Innate immunity is the first line of host defense against invading microorganisms. Innate immune responses to viruses are triggered by recognition of specific structures of diversified pathogens called pathogen-associated molecular patterns (PAMPs). Host cells have multiple defensive mechanisms including pattern recognition receptors (PRRs) that can eliminate viruses through recognition of various viral PAMPs, such as ssRNA and dsRNA produced in virally infected cells. A recent triumph in research into immunity has been the discovery of three families of PRRs: Toll-like receptors (TLRs), Retinoic acid-inducible gene 1 (RIG- I) like helicases (RLRs) and nucleotide-binding domain and leucine-rich-repeat-containing proteins (NLRs). All three families are involved in influenza virus recognition and responses by the host[12] (Figure 1).

RIG- I is a highly inducible cytoplasmic RNA helicase that activates antiviral responses to influenza virus by cell-signal mediated activation of interferon (IFN) production[13,14]. Stimulation of RIG- I activates specific signaling pathways that lead to activation of nuclear factor-κB (NF-κB) which are crucial for inflammatory cytokine induction, and/or induction of interferon regulatory factor 3/7 (IRF3/7) which is important for the IFN-induced antiviral response. Many studies have confirmed that RIG- I regulation during influenza virus infection is important in the antiviral response and for modulation, either directly or indirectly, of proinflammatory cytokine responses[15,16]. We have shown that RIG- I induction is inhibited by cigarette smoking (CS) in our human organ culture model[17]. We demonstrated that 2%-20% cigarette smoke extract (CSE) inhibited influenza-induced RIG- I mRNA and protein expression as well as expression of the anti-viral cytokines interferon g induced protein 10 and IFN-β in human lung.

Of the 13 mammalian TLRs, TLR3 and 7 are the most important PRRs for influenza virus recognition.

The influenza virus ssRNA genome is recognized by TLR7 in plasmacytoid dendritic cells (pDC) in humans[18,19]. Others have shown that CS suppresses key pDC functions upon respiratory syncytial virus (RSV) infection by a mechanism that involves downregulation of TLR7 expression and decreased activation of IRF-7[20]. The effect of CS on TLR7 in influenza virus infected pDC should be similar although it has not been evaluated.

Double-stranded RNA (dsRNA) is produced during viral replication and is recognized by endosomal TLR3[21]. Surprisingly, TLR3-deficient mice appear to be even more resistant to influenza infections than wild type mice, in terms of mortality[22]. Although high viral loads have been detected in the lung, viral load does not appear to underlie disease susceptibility in this model. In *in vitro* studies, CSE enhances rhinovirus-induced TLR3 expression and interleukin-8 secretion in A549 cells[23]. In human bone marrow mononuclear cells, CSE induces TLR2, TLR3 and TLR4 expression[24]. *In vivo*, CS augments the expression and responses of TLR3 in human macrophages[25] and in murine lung tissue. However, CS exacerbated poly(I:C)-induced neutrophilia and airway hyperresponsiveness[26]. Recently, Todt et al have reported that smoking decreased the response of human lung macrophages to dsRNA by reducing TLR3 expression. Alveolar macrophage of smokers show reduced C-X-C motif chemokine 10 production in response to poly(I:C) stimulation *in vitro*[27]. Therefore, CS alone is likely to slightly induce TLR3 expression. However, CS may suppress additional induction of TLR3 by virus. TLR3 is highly expressed in mouse innate immune cells, but shows a low level of expression in human monocytes, macrophages and dendritic cells[28]. This might lead to some conflicting results in studies of TLR3 expression in human and mouse models.

In the NLR family, Sabban *et al*[29] found that nucleotide-binding oligomerization domain-containing protein 2 (NOD2) confers responsiveness to ssRNA in terms of IRF3 activation and IFN-β production. Furthermore, wild-type cells treated with NOD2-specific small interfering RNA or bone marrow-derived macrophages from NOD2-deficient mice failed to produce an antiviral response after transfection with ssRNA, as is contained in RSV and vesicular stomatitis virus. It has been reported that CSE delays NOD2 expression and affects NOD2/receptor-interacting serine-threonine kinase 2 interactions in intestinal epithelial cells[30]. Thus, CS might interfere with the NLR-initiated innate response to influenza virus although further experiments are needed to examine this possibility.

In addition to inhibition of PRRs, CS could also affect the downstream signaling and transcription factors controlling the expression of IFN. For example, expression of IRF7 is critical for amplification of the type I interferon response. The expression of IRF7 was significantly decreased in influenza-infected nasal epithelium from smokers[31]. Furthermore, the data indicated that DNA methylation of the *IRF7* gene and expression of the DNA (cytosine-5-)-methyltransferase1 was enhanced in cells from smokers. Previous studies demonstrated that hypermethylation of IRF7 results in decreased ability of type I IFNs to induce gene expression[32]. In the above report, IRF7 induction after influenza was suppressed both *in vitro* in long-term differentiated cultures of nasal epithelium, and in freshly biopsied nasal epithelial cells obtained from smokers after inoculation with the live-attenuated influenza virus vaccine. Mechanistically, another group found that cigarette smoke-conditioned medium decreased the expression of IRF-7 transcripts and suppressed the nuclear translocation of the key transcription factors, NF-κB and IRF-3, after poly(I:C) stimulation[33].

CS INDUCED CELLULAR OXIDATIVE STRESS AND INFLUENZA INFECTION

CS may affect many physiologic conditions which further alter host defense and virus clearance of lung cells. One of the most important mechanisms of CS-induced alteration is by increasing cellular oxidant stress. CSE contains high concentrations of reactive oxygen species (ROS), nitric oxide, peroxynitrite, and free radicals of organic compounds[34-36]. In addition to these short-lived, highly reactive substances, previous studies have shown that aqueous cigarette tar extracts also contain pro-oxidant substances that increase cellular production of ROS by NADPH oxidases[37-39]. NADPH oxidase-mediated generation of ROS is part of the innate immune defense of phagocytic cells and a variety of non-phagocytic cells against foreign pathogens. Endogenous antioxidant systems cope with the oxidative burden and limit potential toxicity of ROS. However, excess ROS may overwhelm antioxidant capacity and perturb the balance in this reduction-oxidation equilibrium, and damage cells and tissues through oxidative stress. In this regard, ROS are involved in the tissue injury associated with a number of inflammatory diseases, including rheumatoid arthritis[40], ischemia-reperfusion injury[41] and the adult respiratory distress syndrome[42]. Most importantly, mice lacking a functional NADPH oxidase exhibit increased viral clearance, reduced lung damage and improved lung function during influenza virus infection[43]. Human and animal studies show that CS produces generalized endothelial dysfunction[44-46], which is usually an indicator of increased oxidative stress which can be mediated by NADPH oxidases. Thus the increased NADPH oxidase activity induced by CS might play a major role in oxidative stress in human lung and inhibit the innate response to influenza virus (Figure 1).

CS increases the level of oxidants in the lungs, resulting in depletion of antioxidants. In response to CS, pulmonary epithelial cells counteract increased levels of oxidants by activating Nrf2-dependent pathways to augment the expression of detoxification and antioxidant enzymes. Nrf2 is a transcription factor and the Nrf2 antioxidant response pathway is the primary cellular defense

against the cytotoxic effects of oxidative stress. Among other effects, Nrf2 increases the expression of numerous antioxidant and pollutant-detoxifying genes and is essential to protect the lungs from oxidative injury and inflammation. Yageta *et al*[47] have examined the role of Nrf2 in protection against influenza virus-induced pulmonary inflammation after CS exposure with both *in vitro* and *in vivo* approaches. Their data indicate that the antioxidant pathway controlled by Nrf2 is pivotal for protection against the development of influenza virus-induced pulmonary inflammation and injury under oxidative conditions[47]. The results further proved that oxidant stress contributes to CS-mediated susceptibility to influenza infections.

Blake *et al*[48] have developed a novel mouse model in which the cytosolic inhibitor of Nrf2, Keap1, is genetically deleted in Clara cells, which predominate in the upper airways in mice. Deletion of Keap1 in Clara cells resulted in increased expression of Nrf2-dependent genes. Deletion of Keap1 in airway epithelium also protected Clara cells against oxidative stress *ex vivo* and attenuated oxidative stress and CS-induced inflammation *in vivo*[48]. Therefore, current reports suggest that reducing oxidative stress in cells has a potential therapeutic effect, not only restoring virus recognition by PRRs suppressed by CS, but also by decreasing oxidant-mediated inflammation and cellular injury.

Recent data from our laboratory also demonstrated that CS-mediated cellular oxidant stress is the major mechanism of suppression of viral-mediated induction of the major RNA virus sentinel, RIG- I , in human lung[17]. We found that CSE treatment inhibited influenza-induced anti-viral cytokine expression in our human lung organ culture model. This is associated with CSE-inhibited mRNA and protein expression of RIG- I , which is important in the antiviral host response. However, inhibition of viral-mediated RIG- I induction by CSE was prevented and antiviral cytokine responses were restored by the antioxidant, N-acetyl cysteine (NAC). These findings show that CSE suppresses anti-viral responses in influenza virus infected human lung through oxidative inhibition of RIG- I . CS is the major cause of chronic obstructive pulmonary disease (COPD) and exacerbates the susceptibility of the host to respiratory infectious diseases and the attendant pathology[49]. Restoration of these responses by NAC may be an important mechanism for the recent finding that treatment of COPD patients with high-dose NAC resulted in decreased exacerbations[50].

In summary, epidemiological studies suggest that CS is a major risk factor for influenza caused morbidity and mortality. The innate immune system senses influenza virus invasion through recognition of specific ligands by TLR3/7, NLR and cytosolic RIG- I . CS inhibits RIG- I , TLR3 and possibly TLR7 recognition of influenza virus. ROS induced by CS are involved in the interference of PRR function. Reducing oxidative stress in cells, either by using antioxidants or by manipulating Nrf2 overexpression, has a potential therapeutic effect of restoring virus recognition by PRRs suppressed by CS.

More studies will be required to enhance our understanding of the mechanism whereby CS suppresses the human immune system and also of the process that controls influenza virus infection. This will be important in designing strategies for the development of novel treatments to mitigate the adverse consequences of CS and flu infection.

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P- Reviewers: Fujita J, Julio RL, Ryffel B, Yokota S
S- Editor: Song XX **L- Editor**: A **E- Editor**: Liu SQ

World Journal of Immunology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5411/wji.v4.i1.26

World J Immunol 2014 March 27; 4(1): 26-33
ISSN 2219-2824 (online)

MINIREVIEWS

Role of myeloid-derived suppressor cells in autoimmune disease

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Received: October 25, 2013 Revised: December 12, 2013
Accepted: January 17, 2014
Published online: March 27, 2014

Abstract

Myeloid-derived suppressor cells (MDSCs) represent an important class of immunoregulatory cells that can be activated to suppress T cell functions. These MDSCs can inhibit T cell functions through cell surface interactions and the release of soluble mediators. MDSCs accumulate in the inflamed tissues and lymphoid organs of patients with autoimmune diseases. Much of our knowledge of MDSC function has come from studies involving cancer models, however many recent studies have helped to characterize MDSC involvement in autoimmune diseases. MDSCs are a heterogeneous group of immature myeloid cells with a number of different functions for the suppression of T cell responses. However, we have yet to fully understand their contributions to the development and regulation of autoimmune diseases. A number of studies have described beneficial functions of MDSCs during autoimmune diseases, and thus there appears to be a potential role for MDSCs in the treatment of these diseases. Nevertheless, many questions remain as to the activation, differentiation, and inhibitory functions of MDSCs. This review aims to summarize our current knowledge of MDSC subsets and suppressive functions in tissue-specific autoimmune disorders. We also describe the potential of MDSC-based cell therapy for the treatment of autoimmune diseases and note some of hurdles facing the implementation of this therapy.

Key words: Myeloid-derived suppressor cells; Autoimmune disease; Autoimmunity; T cells; Chronic inflammation; Immune regulation

Core tip: Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of cells with immunosuppressive abilities. MDSCs inhibit T cell function and regulate immune responses in cancer and autoimmune diseases. Therapeutic administration of MDSCs in the mouse models of multiple sclerosis, rheumatoid arthritis, and diabetes has shown promising results. Thus, MDSCs have potential in cell-based treatments of autoimmune disorders. However, the role of MDSCs in autoimmunity is complex and not fully understood. Further studies are needed before new therapies can be implemented.

Crook KR, Liu P. Role of myeloid-derived suppressor cells in autoimmune disease. *World J Immunol* 2014; 4(1): 26-33 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i1/26.htm DOI: http://dx.doi.org/10.5411/wji.v4.i1.26

INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells that have the ability to suppress T cell functions[1]. MDSCs are derived from the bone marrow and arise from a delay in maturation during pathologic conditions, such as cancer, chronic inflammation, infection, and traumatic stress[2]. Most studies focus on the pathogenic nature of MDSCs in cancer, where suppression of T cell-mediated immune

responses prevents immune surveillance and clearance of developing tumors[3-5]. Recently, MDSCs have been reported to regulate autoimmunity and control the generation and perpetuation of autoimmune diseases[6]. In this review, we will summarize the current knowledge of MDSC subsets and suppressive functions in tissue-specific autoimmune disorders. We also describe the potential of MDSC-based cell therapy for the treatment of these autoimmune diseases, while noting some of the obstacles that may hinder the implementation of this therapy.

MDSC INVOLVEMENT IN AUTOIMMUNE DISEASES

Our knowledge of the origination and functions of MDSCs has come mainly from studies in tumor models and from cancer patients[1,5,7]. The role of MDSCs in autoimmune diseases is only starting to be elucidated. We now know that MDSCs are involved in a number of different autoimmune disorders, including multiple sclerosis (MS), type 1 diabetes, rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and autoimmune hepatitis. In steady state conditions, MDSCs reside primarily in the bone marrow. Under pathological conditions, MDSC populations expand and can be detected in the spleen, lymph nodes, cancerous tumors, and bloodstream. An early study using a mouse model of autoimmune uveoretinitis showed that the accumulation of nitric oxide-producing monocytes in the choroid and retina of the eye correlated with the severity of disease[8]. A later study showed similar results and confirmed the identity of these cells to be MDSCs[9]. Studies using the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), showed that MDSCs were present in the demyelinated areas of the spinal cord tissue of mice. Another EAE model showed that MDSC accumulation in the spleen correlated with disease progression[10]. Here, they showed that the start of MDSC accumulation occurred during the asymptomatic phase and increased throughout the onset phase. At the peak of the disease, MDSC accumulation reached its highest level, and then began to decrease during the recovery phase and returned to steady state levels by disease resolution. Similar results were found using collagen-induced arthritis (CIA), a mouse model of RA, where MDSC accumulation in the spleen correlated with the course of disease[11]. In humans, MDSCs were found to be enriched in the bloodstream of patients with active MS, but were only slightly elevated in the blood of patients in recovery[12].

MDSCs require certain signals for their expansion and activation. The factors responsible for driving the expansion of MDSCs include cyclooxygenase-2, prostaglandins, interleukin 6 (IL-6), macrophage colony-stimulating factor (M-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF)[9,13-18]. Most of these factors trigger signaling pathways that stimulate the proliferation of myeloid cells in the bone marrow and inhibit their differentiation into mature cells[3]. MDSCs can be activated to suppress T cell functions *via* interferon gamma (IFNγ) and transforming growth factor beta (TGF-β)[13]. Blocking IFNγ production by activated T cells abolishes MDSC-mediated T cell suppression[1,11,19]. Cancer models have identified IL-6, IL-1β, prostaglandin E_2, and the calcium binding proteins S100A8 and S100A9, as factors important for the accumulation of MDSCs at sites of inflammation[17,20,21]. Tumor necrosis factor (TNF) signaling drives MDSC accumulation in the periphery by promoting MDSC survival and inhibiting apoptosis[22]. Treatment with a TNF-α antagonist showed decreased MDSC accumulation in the spleen in response to chronic inflammation[23].

MDSC SUBSETS IN AUTOIMMUNITY

Early classification of MDSCs was based on cell surface expression of CD11b and Gr-1. The $CD11b^+Gr\text{-}1^+$ subgroup is now divided into two separate groups, exhibiting either a monocytic morphology or a granulocytic morphology[24]. Granulocytic MDSCs (G-MDSCs) display a $CD11b^+Ly6C^{low}Ly6G^+$ phenotype, whereas monocytic MDSCs (M-MDSCs) are $CD11b^+Ly6C^+Ly6G^-$[18,24-26]. The two groups also differ in functionality[18,25,27]. MDSCs suppress T cell functions *via* a number of different mechanisms involving the production of soluble mediators or through cell-cell contact[28-31]. G-MDSCs frequently inhibit T cell function through arginase-1 enzyme activity. M-MDSCs more commonly inhibit T cell functions *via* nitric oxide production. IFNγ-mediated activation of MDSCs results in the upregulation of arginase-1 and nitric oxide production. In the CIA model, MDSCs were found to inhibit both T cell proliferation and $CD4^+$ T cell differentiation into Th17 cells[11]. Here, the researchers used the total $CD11b^+Gr\text{-}1^+$ population from the spleen and found both arginase-1 and nitric oxide to be mechanisms of inhibition. The Gr-1 antibody recognizes both Ly6G and Ly6C surface antigens, therefore the population of cells used for their studies contained both G-MDSCs and M-MDSCs. In a mouse model of diabetes, $CD11b^+Gr\text{-}1^+$ cells were found to inhibit $CD8^+$ and $CD4^+$ T cell responses *via* nitric oxide- and IL-10-dependent mechanisms[32]. In the EAE model, G-MDSCs from myelin oligodendrocyte glycoprotein-immunized mice were found to express high levels of programmed cell death 1 ligand 1 (PD-L1), a costimulatory molecule that negatively regulates T cell proliferation. G-MDSCs were found to inhibit autoantigen-priming of Th1 and Th17 cells in a PD-L1-dependent manner[12]. Interestingly, one report showed that $CD11b^+Gr\text{-}1^+$ cells isolated from mice with EAE inhibited T cell proliferation in co-culture but promoted Th17 cell differentiation under Th17-polarizing conditions[33].

M-MDSCs also display immunosuppressive effects during autoimmune diseases. Recent data showed that M-MDSCs induced during the priming phase of EAE were potent suppressors of activated T cells and mediated T cell inhibition through the production of nitric

oxide[18]. Nitric oxide production by MDSCs results in the nitrosylation of cysteine residues, leading to a significant decrease in mRNA stability, and thereby preventing the production of cytokines required for T cell proliferation[28]. Another study demonstrated that activation of M-MDSC suppressive function occurred at the peak of EAE disease[34]. This study determined that the suppression of T cell responses was due to M-MDSC-mediated nitric oxide production. Furthermore, transfer of activated M-MDSCs led to apoptosis of T cells in the central nervous system and decreased EAE severity. In autoimmune arthritis, clinical trials against C-C chemokine receptor 2 (CCR2), the major chemokine receptor mediating monocyte recruitment, were surprisingly unsuccessful as monocytes/macrophages were thought to be pathogenic in RA[35-37]. Interestingly, CCR2-deficient mice are now known to develop exacerbated CIA[38,39]. The underlying mechanisms contributing to the aggravated disease are not clear. However, our data showed that M-MDSCs were absent from the periphery of collagen-immunized CCR2-deficient mice, as CCR2 is required for the emigration of M-MDSCs from the bone marrow[38,40]. Further, M-MDSCs isolated from the bone marrow of CCR2-deficient mice with CIA inhibited $CD4^+$ T cell proliferation and mitigated CIA severity, suggesting M-MDSCs are required for the regulation of autoimmune arthritis[41].

Human MDSCs are identified as $CD14^+CD16^+$ and $CD14^+CD16^-$ cells. These $CD14^+$ cells were found to be abundant in the blood and synovial fluid of RA patients[42,43]. Recently, MDSCs were shown to mediate enhancement of regulatory T cell (Treg) suppressive functions[43]. Here, Tregs were isolated from healthy subjects and their suppressive activity and cytokine expression were analyzed after co-culture with $CD14^+$ cells. Results showed an increase in the expression of IFNγ, TNF-α, IL-17, and IL-10 by Tregs, a sustained Treg phenotype, and an enhanced capacity to suppress T cell-mediated proinflammatory cytokine production and T cell proliferation.

Taken together, these studies demonstrate that MDSCs can use various functions to suppress T cell responses and suggest that MDSC differentiation and function may be influenced by the distinct environment associated with each type of disease. Although both G-MDSCs and M-MDSCs can suppress T cell functions, further research is needed to confirm whether the two subsets have different outcomes in different diseases (Table 1).

MDSC-MEDIATED SUPPRESSION OF ANTIGEN-SPECIFIC IMMUNE RESPONSES

Loss of immunological tolerance is the basis for the development of autoimmune diseases. Recognition of self-antigens leads to autoimmune-driven tissue inflammation. However, regulation of the responses to self-antigens must be highly specific in order for the host immune recognition of pathogens to remain intact. MDSCs may play a crucial role in maintaining this balance as they are capable of suppressing antigen-specific immune responses. It is believed that MDSCs internalize antigens and present them to T cells, bringing the two cells into close contact. Peroxynitrite, a derivative of nitric oxide, causes nitration of tyrosine residues on the T cell receptor (TCR), thereby preventing binding between the major histocompatibility complex (MHC) and peptide[44]. Increased levels of nitrotyrosine have been documented for patients suffering from MS, RA, autoimmune myocarditis, and diabetes[45-48]. In a cancer model, increased production of peroxynitrite and hydrogen peroxide resulted from the interaction between immature myeloid cells and antigen-specific $CD8^+$ T cells in the presence of the specific antigen, but not in the presence of the control antigen[29]. In some cancer models, arginase-1 production is the mechanism of MDSC-mediated suppression[31,49]. The arginase-1 enzyme hydrolyzes arginine, depleting the pool of arginine available to the cell[50-52]. A deficiency in arginine prevents the formation of CD3 molecules[53]. The absence of CD3 prevents signaling through the TCR upon recognition of a specific antigen-MHC complex.

In one study of autoimmune diabetes, MDSCs induced the antigen-specific expansion of Tregs, which resulted in the suppression of T cell proliferation and prevented the onset of disease[54]. The authors described that MDSC-mediated expansion of Tregs was dependent on antigen presentation by MHC class Ⅱ molecules. For these experiments, hemagglutinin (HA)-specific $CD4^+$ T cells were adoptively transferred to mice, followed by the administration of MDSCs and HA antigen. The results showed a significant reduction in disease upon administration of MDSCs and HA, but no decrease in disease when MDSCs were administered with the ovalbumin peptide, confirming that the MDSC-mediated suppression was antigen-specific.

MDSCs also mediate suppression of non-specific T cell responses, *i.e.,* mitogen-activated T cell responses, suggesting MDSCs may be involved in the late phase of tissue inflammation during autoimmune diseases. Others have hypothesized that MDSCs function in both antigen-specific and non-specific manners depending on the signals they are exposed to in a particular microenvironment[55]. Indeed, comparison of MDSCs isolated from the spleen to those isolated from a tumor showed that splenic MDSCs were able to inhibit antigen-specific T cell responses *via* the production of reactive oxygen species, whereas MDSCs isolated from the tumor inhibited T cells nonspecifically and more potently than those from the spleen[56]. T cells isolated from the peripheral lymphoid organs of human cancer patients, or from a mouse tumor model, are still responsive to non-cancer related stimuli, including viruses, IL-2, and anti-CD3/CD28 antibodies[1,57]. This suggests that the expansion of MDSCs does not induce systemic immune suppression. Taken together, these data suggest that MDSCs from the site of inflammation may be more potent and far-reaching

Table 1 Myeloid-derived suppressor cells in autoimmune disease models

Human disease	Mouse model	Phenotype	T cell suppression	Suppressive mechanism	Suppressive role *in vivo*
Multiple sclerosis	EAE	$CD11b^+Ly6C^{high}$ (M-MDSCs)	$CD4^+$ T cells	NO-apoptosis	Not determined[18]
	EAE	$CD11b^+Ly6G^-$ (M-MDSCs)	$CD4^+$, $CD8^+$, Ag-specific $CD4^+$ T cells	NOS	No effect by naïve MDSCs[77]
	EAE	$CD11b^+Ly6C^{high}$ (M-MDSCs)	Not determined	Not determined	Increase severity[73]
	EAE	$Arg\text{-}1^+CD11b^+Gr\text{-}1^{low}$ (M-MDSCs)	$CD3^+$ T cells	Apoptosis	Not determined[10]
	EAE	$CD11b^+Ly6C^+$ (M-MDSCs)	$CD4^+$ T cells	NO	Reduce severity by late phase MDSCs[34]
	EAE	$CD11b^{high}Ly6G^+Ly6C^-$ (G-MDSCs)	Th1 and Th17 cells	PD-L1	Reduce severity[12]
	EAE	$CD11b^+Gr\text{-}1^+$	Promote Th17 cells	IL-1β	Increase severity[33]
	EAE	$CD11b^+Gr\text{-}1^+$	Ag-specific Th17 cells	iNOS, arginase-1 and IL-10	Ablated iNKT-induced disease mitigation[78]
Rheumatoid arthritis	CIA	$CD11b^+Ly6C^+Ly6G^-$ (M-MDSC)	$CD4^+$ T cells	NO	Reduce severity[41]
	CIA	$CD11b^+Gr\text{-}1^+$	Th17 cells	Arginase and iNOS	Reduce severity[11]
	Proteoglycan-induced arthritis	$CD11b^+Gr\text{-}1^+$	Ag-specific T cells	NO and ROS	Not determined[79]
Systemic lupus erythematosus	$MRL\text{-}fas^{lpr}$	$CD11b^+Gr\text{-}1^{low}$ (M-MDSCs)	$CD4^+$ T cells	Arginase-1	Not determined[80]
Inflammatory bowel disease	HA-transgenic mice	$CD11b^+Gr\text{-}1^+$	Ag-specific $CD8^+$ T cells	NO-apoptosis	Reduce severity[14]
	DDS-induced colitis	$CD11b^+Gr\text{-}1^+$	Not determined	Not determined	Reduce severity[75]
	IL-10-/-	$CD11b^+Gr\text{-}1^+$	MLN T cells	Not determined	Not determined[81]
	TNBS-induced colitis	$CD11b^+Gr\text{-}1^+$	Splenocytes	Not determined	Reduce severity[74]
T1D	INS-HA/RAG-/-h-CD20/NOD	$Gr\text{-}1^+CD115^+$ (M-MDSCs)	Induce Tregs and inhibit Teff cells	TGF-β and IL-10	Reduce severity[54]
		$CD11b^+Gr\text{-}1^+$	$CD4^+$ and $CD8^+$ T cells induce Tregs	NO and IL-10	Not determined[32]
Autoimmune hepatitis	Tgfb-/-	$CD11b^+Ly6C^{high}$ $Ly6G^-$ (M-MDSCs)	$CD4^+$ T cells	NO	Not determined[82]
Inflammatory eye disease	EAU	$CD11b^+Gr\text{-}1^+$ $Ly6G^-$ (M-MDSCs)	$CD4^+$ T cells	TNFR-dependent, Arginase	Not Determined[9]
	EAU	REP-induced $CD11b^+Gr\text{-}1^+$	$CD4^+$ T cells	Not determined	Reduce severity[76]
Alopecia areata	Alopecia areata-eczema	$CD11b^+Gr\text{-}1^+$	$CD4^+$ and $CD8^+$ T cells	CD3-zeta down-regulation	Local MDSC administration reduces severity[83]

TID: Type 1 diabetes; EAE: Experimental autoimmune encephalomyelitis; CIA: Collagen-induced arthritis; MLR: Murphy roths large; HA: Hemagglutinin; DDS: Dextran sulphate sodium; INS: Insulin; RAG: Recombination-activating gene; NOD: Non-obese diabetic; EAU: Experimental autoimmune uveitis; M-MDSCs: Monocytic myeloid-derived suppressor cells; G-MDSCs: Granulocytic myeloid-derived suppressor cells; MLN: Mesenteric lymph node; NO: Nitric oxide; NOS: Nitric oxide synthase; PD-L1: Programmed cell death 1 ligand 1; iNOS: Inducible nitric oxide synthase; ROS: Reactive oxygen species; IL-10: Interleukin 10; TGF-β: Transforming growth factor beta; IL-1β: Interleukin 1 beta; TNFR: Tumor necrosis factor receptor.

in their suppressive effects than those MDSCs in the peripheral organs. The MDSCs in circulation may function to prevent the spread of inflammation to other areas of the body, without compromising immune recognition of pathogens.

THERAPEUTIC POTENTIAL OF MDSC-BASED TREATMENTS

Therapeutic approaches involving MDSCs require their purification and/or proliferation *in vitro*. MDSCs migrate to peripheral lymphoid organs where they differentiate into granulocytes, monocytes/macrophages, and dendritic cells (DCs). GM-CSF has been shown to drive MDSC accumulation at sites of inflammation[58,59] and has been used to generate MDSCs from bone marrow cells *in vitro*[60]. However, the concentration of GM-CSF in the media must be tightly regulated as different concentrations of GM-CSF may lead to the generation of neutrophils or DCs[60,61]. Vascular endothelial growth factor (VEGF) is important in the differentiation of hematopoietic progenitor cells[62], and studies have shown that blocking VEGF binding leads to increased differentiation of MDSCs into DCs[63]. Similar results were shown for stem cell factor, where blocking its function led to reduced MDSC expansion[64]. Factors such as granulocyte colony-stimulating factor (G-CSF) and M-CSF are also known to induce MDSC expansion. G-CSF induces the proliferation of G-MDSCs *via* the Janus kinase/signal transducers and activators of transcription pathway (Jak/STAT)[65]. In the presence of IL-6, M-CSF was shown to inhibit DC generation from hematopoietic stem cells (HSCs), thereby redirecting HSC differentiation towards MDSCs[66].

The calcium binding proteins, S100A8 and S100A9, are upregulated in some autoimmune conditions, including RA, MS, and IBD[66-68]. These proteins are secreted by MDSCs[69] and may work in an autocrine fashion to promote the accumulation of MDSCs while simultaneously preventing their differentiation into DCs[70]. MDSC generation, expansion, and gain of specific suppressive abilities occur primarily under inflammatory conditions such as infection, cancer, trauma, and autoimmune diseases. It is important to note that MDSCs are not terminally differentiated, and thus may mature into antigen-presenting cells, such as macrophages or DCs, highlighting a potential complication for therapeutic attempts. Therefore, in order to develop effective MDSC-based therapies, we must first understand how different cell types respond to different inflammatory mediators and determine how these inflammatory mediators affect the potency and/or suppressive mechanisms of MDSCs.

A number of studies have provided insight into the use of MDSCs for treatment of autoimmune diseases. In a murine model of diabetes, MDSCs were generated *in vitro* by culturing hepatic stellate cells with DCs[71]. This method of MDSC generation was previously shown to produce highly suppressive cells in an IFNγ-dependent manner[72]. In the diabetes study, these *in vitro*-generated MDSCs were mixed with pancreatic islet cells and transplanted into diabetic mice. The MDSCs induced Treg expansion in the allograft site, resulting in the inhibition of $CD8^+$ T cell responses[71]. In a mouse model of IBD, MDSCs were found to be upregulated in the spleen and intestine of IBD mice[14]. Further data showed that these MDSCs effectively prevented T cell proliferation and induced T cell apoptosis after transfer of $CD8^+$ T cells[14]. One report showed that the *in vivo* transfer of G-MDSCs in the EAE model resulted in the delayed onset of disease and a significant reduction in demyelination[12], however other studies were not as successful[33,73]. Adoptive transfer of MDSCs also led to reduced disease severity in models of RA[11,41], IBD[74,75], and inflammatory eye disease[76].

CONCLUSION

MDSCs represent an important class of immunoregulatory cells. MDSCs display particular heterogeneity and plasticity, and for these reasons they have become an attractive candidate for the treatment of autoimmune diseases. On the other hand, MDSCs are very difficult to work with because of their diverse nature. MDSCs have multiple phenotypes which inhibit T cell responses by multiple mechanisms, and their environment dictates the development of suppressive properties and activation pathways. Additionally, the maturation/differentiation of these cells may depend on the particular inflammatory signals received from their microenvironment. Though MDSCs hold promise in the treatment of autoimmune diseases, their full utilization is stalled by our limited understanding of their phenotype, differentiation, cellular functions, and influence on the microenvironment.

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P- Reviewers: Sotelo J, Yves R **S- Editor**: Song XX
L- Editor: A **E- Editor**: Liu SQ

World Journal of Immunology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5411/wji.v4.i1.34

World J Immunol 2014 March 27; 4(1): 34-41
ISSN 2219-2824 (online)

MINIREVIEWS

Immune thrombocytopenia in adults

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Received: September 27, 2013 Revised: November 21, 2013
Accepted: December 13, 2013
Published online: March 27, 2014

Core tip: In this manuscript we evaluate all aspects of immune thrombocytopenia (ITP). We outline the etiology, pathogenesis, diagnosis and treatment of ITP. We describe the first and second-line therapies in detail. Also, the mechanism of the actions of drugs is described.

Kurtoğlu E, Karakuş V. Immune thrombocytopenia in adults. *World J Immunol* 2014; 4(1): 34-41 Available from: URL: http://www.wjgnet.com/2219-2824/full/v4/i1/34.htm DOI: http://dx.doi.org/10.5411/wji.v4.i1.34

Abstract

Immune thrombocytopenia is an autoimmune disease resulting in the destruction of platelets. It is classified as acute, thrombocytopenia occurring for < 6 mo and usually resolving spontaneously, and chronic, lasting > 6 mo and requiring therapy to improve the thrombocytopenia. The underlying defects leading to autoantibody production are unknown. Molecular mimicry appears to play a role in the development of self-reactive platelet antibodies after vaccination and certain viral infections. Platelet life span is reduced as a consequence of antibody-mediated clearance by tissue macrophages in essentially all patients. Diagnosis is based on the exclusion of the other causes of thrombocytopenia. Steroid is the first choice of the treatment, often followed by splenectomy in unresponsive cases. Intravenous immunoglobulin, anti-Rho(D) immune globulin, azathioprine, cyclosporine A, cyclophosphamide, danazol, dapsone, mycophenolate mofetil, rituximab, thrombopoietin receptor agonists and vinca alkaloids are other choices of treatment.

Key words: Immune thrombocytopenia; Splenectomy; Intravenous immunoglobulin; Autoimmune thrombocytopenia

INTRODUCTION

Immune thrombocytopenia (ITP) is an autoimmune disease involving antibody and cell-mediated destruction of platelets and suppression of platelet production that may predispose to bleeding which may be even fatal. Recent recommendations from an international working group suggest that ITP be used to designate all cases of immune-mediated thrombocytopenia, whether occurring as a component of another clinically evident disorder or drug exposure, secondary ITP or, in the absence of a clear predisposing etiology, primary ITP[1,2].

The international working group also recommends that a platelet count below 100×10^9/L, rather than 150×10^9/L, be required for diagnosis. This threshold is based on observational evidence that fewer than 10% of otherwise healthy individuals with a stable platelet count between 100 and 150×10^9/L develop more severe unexplained ITP over the ensuing 10 years. This review focuses on primary ITP in the adult population but includes certain aspects of secondary forms and pediatric ITP where pertinent[3].

INCIDENCE AND PREVALENCE

The annual incidence of ITP in the United States is estimated to be 1.6/100000. Acute ITP, defined as thrombocytopenia occurring for < 6 mo and usually resolving

spontaneously, most often affects children and young adults. The incidence peaks in the winter and spring, following viral infections. Acute ITP is most common between 2 and 6 years of age. Approximately 7% to 28% of children with acute ITP develop the chronic form. Chronic ITP, lasting > 6 mo and requiring therapy to improve the thrombocytopenia, occurs most commonly in adults, as emphasized in the oldest reported series in the literature. In the reported series, both acute and chronic ITP cases were reviewed, 67% of 271 patients and 45% of 737 patients were below 21 and 15 years of age, respectively. In chronic ITP in adults, the median age is usually 40 to 45 years, although in one large series, 74% of 934 cases were younger than the of age 40. The ratio of female to male is nearly 1:1 in acute ITP and 2 to 3:1 in chronic ITP[4].

Estimates of the incidence of adult-onset ITP range from approximately 1.6 to 3.9 per 100000 persons per year, with a prevalence ranging from 9.5 to 23.6 per 100000 persons, based on diagnostic codes in the United Kingdom health registry[5,6]. Estimates based on the International Classification of Diseases, 9th revision codes at hospital discharge in the United States are somewhat lower[7]. However, in light of the vagaries of diagnosis and diagnostic coding, as well as the likelihood that some affected patients may not seek medical attention, the actual frequency of ITP and the number of individuals requiring therapy is uncertain.

ETIOLOGY

The underlying defects leading to autoantibody production are unknown. Heritability is uncommon, although predisposing polymorphisms in cytokines and Fcγ receptors have been described. A Th1/Th0 cytokine profile, a reduction in suppressor T-regulatory cells, and an increase in B-cell-activating factor may predispose to emergence of autoantibodies in response to exogenous antigens. Molecular mimicry appears to play a role in the development of self-reactive platelet antibodies after vaccination and certain viral infections[8-12]. Thrombocytopenia can be caused by a myriad of conditions, including systemic disease, infection, drugs and primary hematological disorders (Table 1)[2].

PATHOGENESIS

Platelet life span is reduced as a consequence of antibody-mediated clearance by tissue macrophages in essentially all patients. Accumulating evidence from studies of platelet kinetics also points to the contribution of immune-mediated suppression of megakaryocyte and platelet development in many patients; megakaryocyte apoptosis and suppression of megakaryopoiesis *in vitro* by ITP plasma/immunoglobulin G (IgG) or T-cells, and responsiveness to thrombopoietin receptor agonists (TRAs)[13-19]. Platelet-reactive antibodies are not detected in all individuals with ITP and a subset of patients do not respond to pharmacological or surgical inhibition of antibody-mediated platelet clearance or B-cell suppression, suggesting the possible involvement of other pathogenic mechanisms such as antibody-mediated apoptosis, antigen shedding and T-cell mediated platelet destruction or marrow suppression[20].

Table 1 Frequent examples of differential diagnosis of immune thrombocytopenia and potential alternative causes of thrombocytopenia identified by patient history

Previously diagnosed or possible high risk of conditions that may be associated with autoimmune thrombocytopenia, for example, HIV, HCV or other infection; other autoimmune/immunodeficiency disorders (including systemic lupus erythematosus; malignancy (*e.g.*, lymphoproliferative disorders); recent vaccination
Liver disease (including alcoholic liver cirrhosis)
Drugs (prescription or non-prescription), alcohol abuse, consumption of quinine, tonic water, exposure to environmental toxins
Bone marrow diseases including myelodysplastic syndromes, leukemias, other malignancies, fibrosis, aplastic anemia and megaloblastic anemia
Recent transfusions (possibility of post-transfusion purpura) and recent immunizations
Inherited thrombocytopenia: thrombocytopenia-absent radius syndrome, radioulnar synostosis, congenital amegakaryocytic thrombocytopenia, Wiskott-Aldrich syndrome, MYH9-related disease, Bernard-Soulier syndrome, type Ⅱ B von Willebrand disease

HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; **MYH9**: Myosin heavy chain 9.

Although the initial inciting event resulting in provocation of antiplatelet antibodies remains unknown, platelet autoantibodies are often present by the time of diagnosis. Macrophages and dendritic cells of the reticuloendothelial system function to phagocytose circulating antibody-bound antigens, including antibody-targeted platelets. Opsonization of antibody-platelet complexes facilitates intracellular processing of platelets and can lead to presentation by T cells *via* major histocompatibility complex (MHC) Ⅱ as an array of "foreign" platelet peptides. Presentation of platelet peptides by MHC Ⅱ in a stimulatory context activates T cells, leading to enhancement of the antiplatelet immune response and the possibility of epitope spread to additional platelet antigens[21].

In patients with ITP, autoantibodies frequently appear to be directed against Gp Ⅰb/Ⅸ and GP Ⅱb/Ⅲa, although specificity for other platelet antigens can occur. Although antiplatelet autoantibodies appear to play a central role in the pathogenesis of ITP, some patients have no detectable antibodies at the time of diagnosis. This may be explained by limitations inherent to laboratory testing methods and the biology of ITP: Brisk clearance of some types of antibody-platelet complexes may reduce circulating antiplatelet antibody titers to below the threshold of detection; tightly bound antiplatelet antibodies may be difficult to dissociate for study; antibodies with specificity to minor or cryptic antigens on platelets or antigens that reside primarily on megakaryocytes may be missed; and there may simply be a subset of patients in which antiplatelet antibodies are not present. Therefore, although the majority of ITP patients present with features consistent with antibody-mediated autoimmunity

as a central feature of their disease, there exists considerable heterogeneity in the types, titers and likely biology of antiplatelet antibodies in ITP[22,23].

As discussed in more detail in a recent review, the presentation of secondary ITP is often more complex than primary ITP. Similar to the antiplatelet antibodies provoked during *Helicobacter pylori* (*H. pylori)* infection, human immunodeficiency virus (HIV) can provoke anti-HIV antibodies that cross-react with platelet glycoproteins and form immune complexes, as hepatitis C virus (HCV) does. Additional mechanisms of platelet destruction also become apparent from studies in virus-associated ITP[24]. In both HIV and HCV, suppression of viral replication can result in improvement in thrombocytopenia. Interestingly, HIV-associated ITP tends to occur early in HIV infection, whereas non-ITP tends to predominate in more advanced HIV when the immune system has suffered from greater effects of the infection. One possible explanation is that the immune system of HIV patients is more capable of developing autoimmunity in the earlier phases of the disease.

An acute infectious event has long been suspected to be a trigger in the initiation of primary ITP. Acute infection remains a plausible candidate to induce ITP either by providing an opportunity for molecular mimicry or similar targeting of the immune system to platelets or by the mere presence of an acute inflammatory response tipping the balance in a predisposed patient to break tolerance[21].

Patients with systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), antiphospholipid antibody syndrome and rheumatoid arthritis, are prone to developing ITP. A diagnosis of secondary ITP in these patients is complex because non-ITP due to underlying disease or related therapies is also common. These observations are consistent with the notion that a patient with one autoimmune disease is at high risk of developing a second. The mechanisms underlying the development of many autoimmune disorders, including ITP, is unknown. It may also be that during the immune dysregulation leading to autoimmunity to one self-antigen, there is a risk of immune presentation of other self-antigens. Interestingly, many of the features of immune dysregulation described in ITP, such as the shift in Th1/Th2 balance, increased Th17 and altered Treg profiles described above, are also common to other autoimmune diseases[25].

DIAGNOSIS

Personal history, with special attention to drugs and medical conditions that could cause thrombocytopenia, is very important. With a family history, ITP may occasionally be mistaken for an inherited cause of thrombocytopenia. The presence of the latter can often be confirmed by review of the peripheral blood film of the patient as well as other family members with thrombocytopenia. ITP is generally not considered to be an inherited disorder, although some HLA alleles may be more prevalent in ITP patients[26].

Physical examination should be normal aside from bleeding manifestations. Mild splenomegaly may be found in younger patients, but moderate or massive splenomegaly suggests an alternative cause. Constitutional symptoms, such as fever or weight loss, hepatomegaly or lymphadenopathy, might indicate an underlying disorder such as HIV, SLE or a lymphoproliferative disease[27].

ITP is characterized by isolated thrombocytopenia with an otherwise normal complete blood count. Anemia from blood loss may be present but it should be proportional to the amount and duration of bleeding and may result in iron deficiency[2].

In ITP, the peripheral blood smear should appear normal except for the presence of thrombocytopenia, although platelets may be mildly enlarged in some individuals. Both red cell and leukocyte morphologies are normal[26].

Bone marrow examination may be informative in patients older than 60 years of age, in those with systemic symptoms or abnormal signs, or in some cases in which splenectomy is considered. Both bone marrow aspirate and biopsy should be performed. In addition to the morphological assessment, flow cytometry and cytogenetic testing should be considered (evidence level Ⅱb-Ⅳ). Flow cytometry may be particularly helpful in identifying patients with ITP secondary to chronic lymphocytic leukemia[2,27].

International guidelines suggest that testing for reduced immunoglobulin levels and HIV, HCV and *H. pylori* infections should also be considered. Testing for antiphospholipid antibodies, antinuclear antibodies, parvovirus and cytomegalovirus may also be indicated in specific individuals. Testing for antiplatelet antibodies is not commonly performed in the current era because of its relatively low sensitivity and specificity[26].

MANAGEMENT OF ADULT ITP

Relevant factors that contribute to management decisions include the extent of bleeding, comorbidities predisposition to bleeding, complications of specific therapies, activity and lifestyle, tolerance of side effects, potential interventions that may cause bleeding, accessibility of care, patient expectations, patient's worry or anxiety about disease burden, and patient's need for non-ITP medications that may create a bleeding risk[28,29].

Treatment is rarely indicated in patients with platelet counts above 50×10^9/L in the absence of the following: Bleeding due to platelet dysfunction or another hemostatic defect, trauma, surgery, clearly identified comorbidities for bleeding, mandated anticoagulation therapy, or in persons whose profession or lifestyle predisposes them to trauma. Patient's preference must also be considered when discussing treatment options[30].

FIRST-LINE TREATMENT

First-line therapies for ITP include corticosteroids, intravenous immunoglobulin (IVIg) and anti-Rho(D) immune globulin[2].

Table 2 Summary of dosage and toxicity of drugs

Agent	Typical dosing	Time to response	Selected toxicities
Prednis(ol)one	0.5-2 mg/kg per day 2-4 wk followed by slow taper	Several days to several weeks	Mood swings, insomnia, anxiety, psychosis, weight gain, cushingoid facies, hyperglycemia, decreased bone density, hypertension, skin changes, gastrointestinal distress and ulceration, avascular necrosis, increased susceptibility to infections, cataracts, adrenal insufficiency
Methylprednisolone	30 mg/kg per day 7 d	2-7 d	
Dexamethasone	40 mg/d for 4 d every 2-4 wk for 1-4 cycles	Several days to several weeks	
IVIg	0.4 g/kg per day 5 d or 1 g/kg per day 1-2 d	1-4 d	Headache, aseptic meningitis, renal insufficiency, fever, chills, nausea, thromboembolism, anaphylactoid reactions in patients with IgA-deficiency
Anti-Rh(D)	50-75 mcg/kg	1-5 d	Hemolytic anemia, fever, chills. Rarely, intravascular hemolysis, DIC, and renal failure
Splenectomy	N/A	0-24 d	Adverse effects of surgery and anesthesia, increased risk of infection, long-term vascular complications
Rituximab	375 mg/m^2 weekly 4 wk (lower doses may be effective)	1-8 wk	Infusion reactions, reactivation of hepatitis B infection, rare cases of progressive multifocal leukoencephalopathy
Eltrombopag	12.5-75 mg PO daily	1-4 wk	Increased bone marrow reticulin, rebound thrombocytopenia, thrombosis, eltrombopag also associated with liver function test abnormalities
Romiplostim	(1-10 mcg/ kg) SC weekly	1-4 wk	
Azathioprine	1-2 mg/kg per day (maximum 150 mg day)	1-4 wk	Liver function abnormalities, neutropenia, anemia, infection
Cyclosporine	5 mg/kg per day 6 d, then 2.5-3 mg/kg per day (titrated to blood levels of 100-200 ng/mL)	1-4 wk	Renal failure, hypertension, tremor, infection
Cyclophosphamide	1-2 mg/kg PO daily or 0.3-1 g/m^2 *iv* every 2-4 wk 1-3 doses	1-4 wk	Myelosuppression, infection, secondary malignancy
Danazol	200 mg 2-4 times per day	1-4 wk	Acne, hirsutism, dyslipidemia, amenorrhea, liver function abnormalities
Dapsone	75-100 mg daily	1-4 wk	Hemolytic anemia in patients with G6PD deficiency, rash, methemoglobinemia
Mycophenolate mofetil	1000 mg twice daily	1-4 wk	Headache, back pain, infection
Vincristine	1-2 mg *iv* weekly (total dose 6 mg)	1-4 wk	Neuropathy, constipation, cytopenias, thrombophlebitis at the infusion site

IVIg: Intravenous immunoglobulin; DIC: Disseminated intravascular coagulation; PO: Per oral.

Corticosteroids

Standard prednisone therapy, 1 to 2 mg/kg per day, is given until a response is seen and then tapered. Some maintain therapy for an additional week before tapering. There are no guidelines about how to taper: Some decrease the dosage by 50% per week, although many recommend going more slowly, particularly at the lower range of dosing. Up to 85% of patients achieve a clinical response, usually within 7 to 10 d, with platelet counts peaking in 2 to 4 wk. Unfortunately, only about 15% of patients maintain the response over the subsequent 6 to 12 mo. Restarting prednisone often initiates a vicious circle and makes patients vulnerable to steroid toxicities (Table 2)[26].

Pulse dexamethasone therapy consists of 40 mg/d for 4 d for one to three cycles (Dexamethasone 1 mg is equivalent to about 10 mg of prednisone). Pulse dexamethasone therapy as an initial approach to ITP has been developed during the past decade and has been used primarily in research studies. This regimen evolved from studies of patients with multiple myelomas and has the potential to induce more durable remissions in some patients with newly diagnosed ITP. However, high-dose corticosteroids may be associated with increased toxicity, at least in the short term, and should be used cautiously[2].

IVIg

Another primary therapy for ITP is IVIg 0.4 g/kg per day for 5 d or infusions of 1 g/kg per day for 1-2 d[2]. IVIg is associated with numerous adverse effects, including thrombosis, renal insufficiency, headache and anaphylaxis in IgA-deficient patients. It also converts the direct antiglobulin test to positive. IVIg is expensive, inconvenient to administer, and may require lengthy infusions depending on the formulation.

Although IVIg is not a good long-term therapy, it can help raise the platelet count relatively quickly in patients who present with severe thrombocytopenia accompanied by bleeding. Such patients should be treated with high-dose steroids, IVIg and platelet transfusions. IVIg may also be useful to increase platelet counts prior to interventional procedures[26].

Platelet clearance in ITP mediated by most anti-GPIb antibodies may occur through an Fc-independent process, likely *via* a system that evolved for our innate immunity and for clearance of senescent cells. This type of ITP may not be sensitive to IVIg and other therapies designed based on Fc receptor blockage[31].

Antibody Fc-independent phagocytosis has also been well described in mammals, including Fc-independent opsonization by antibodies[32], as well as antibody and Fc receptor-independent phagocytosis of microbes and other senescent cells[33-36]. In the absence of antibody, specific ligands from bacteria, other foreign microorganisms or the host's senescent cells, may engage receptors directly on phagocytes, such as scavenger receptors, phosphatidylserine counter-receptors, V integrins, com-

plement receptors or C-type lectins[33-38]. In some cases, this engagement can be enhanced by F(ab')2 fragments of antibodies or non-antibody opsonins. These Fc-absent antibodies may bind to receptors on phagocytic cells (*e.g.*, scavenger receptors) or their ligands and induce changes in conformation and affinity of these molecules, which facilitate phagocytosis[32]. Thus, by directly engaging the target, phagocytosis without the need for antibody is an effective mechanism for clearance of microorganisms and senescent cells.

It was demonstrated that the removal of the Fc region of anti-GPIb monoclonal antibodies did not affect the ability of these antibodies to induce thrombocytopenia [*i.e.*, the F(ab')2 portions were as effective as intact antibodies in inducing platelet clearance]. However, when the Fc region of anti-GP Ⅱ bⅢa antibodies was removed, thrombocytopenia was not induced in the same animal model[39].

Intravenous anti-D

An alternative to IVIg for Rh(D)-positive patients before splenectomy is anti-D Ig. At doses of 75 μg/kg, anti-D may increase the platelet count more rapidly compared with the standard dose of 50 μg/kg. Subcutaneous anti-D has been administered to a few patients suffering from chronic ITP who appeared to have the same response rate as those treated with intravenous delivery without relevant side effects. Evidence of hemolysis is present in most patients treated with anti-D. While the decline in hemoglobin concentration rarely exceeds 2 g/dL, several cases of massive intravascular hemolysis and disseminated intravascular coagulation have been reported. Elderly patients, above 65 years of age, with a coexisting infection, autoimmune hemolytic anemia (Evans syndrome), autoimmune disorders or lymphoproliferative disorders appear to be more susceptible to these complications[40].

Platelet transfusions with or without IVIg

Platelet transfusion increases the post-transfusion platelet count by more than 20×10^9/L in 42% of bleeding ITP patients and may reduce bleeding. In a retrospective study of 40 patients, concurrent administration of platelet transfusions and IVIg was associated with resolution of bleeding, rapid restoration of adequate platelet counts and minimal side effects[40,41].

Antifibrinolytics

Antifibrinolytic agents, such as oral or Ⅳ tranexamic acid and epsilon-aminocaproic acid, may be useful in preventing recurrent bleeding in patients with severe thrombocytopenia; however, the efficacy has not been evaluated by randomized trials in ITP patients. Tranexamic acid (1 g, 3 times daily orally) and epsilon-aminocaproic acid (1-4 g every 4-6 h maximum dose, 24 g/d) may be of special value in certain dental or surgical procedures[40-42].

SECOND-LINE TREATMENT

Second-line therapies, as designated by the international working group, include azathioprine, cyclosporine A, cyclophosphamide, danazol, dapsone, mycophenolate mofetil, rituximab, splenectomy, TRAs and vinca alkaloids. The evidence for efficacy of the cytotoxic agents, *i.e.*, cyclophosphamide, the vinca alkaloids and azathioprine, comes from small, non-randomized studies[30]. Although these agents are useful in some patients, they may be associated with significant toxicities and are used less commonly than in the past (Table 2)[26,42].

Splenectomy

Splenectomy probably offers the best response of any treatment for ITP. About 80% of patients with ITP respond rapidly, often within 1 wk. Of those, 15% relapse within the first year and after 10 years, two-thirds remain in remission[43,44].

Splenectomy increases the risk of subsequent infection by encapsulated organisms and patients should be immunized with pneumococcal, *Haemophilus influenzae type B* and meningococcal vaccines, preferably at least 3 wk before the spleen is removed. Splenectomy is associated with pulmonary hypertension and thrombosis, primarily in patients who have had their spleens removed because of accelerated red cell destruction. Whether these risks are applicable to patients with ITP is unknown but if so, they are probably much lower than in patients with red cell disorders[26].

Rituximab

Rituximab, an anti-CD20 monoclonal antibody, has produced variable objective responses. Rituximab causes selective B-cell lysis *in vitro* and B-cell depletion *in vivo*. Involved mechanisms of action include apoptosis, antibody-dependent cytotoxicity. Recovery of B-cell counts usually occurs by 6 to 12 mo after completion of treatment[45].

Several publications have reported the use of rituximab in ITP patients since previous consensus documents were issued and suggest that about 60% of patients respond, with approximately 40% achieving complete response. Responses generally occur after 1 to 2 wk to 6 to 8 wk and last from 2 mo in partial responders to 5 years or longer in 15% to 20% of initially treated patients. Most patients with a durable (> 1 year) complete response will respond to repeat treatment if they relapse[46-48].

Romiplostim

Romiplostim is a peptibody (comprising of an IgG Fc region and four peptidomimetics regions that interact with the thrombopoietin receptor, c-mpl) that is given subcutaneously once a week. Romiplostim performed well in several phase I clinical trials. In a 24 wk phase Ⅲ trial that compared romiplostim against placebo in patients with ITP that had been refractory to other primary treatments, 79% of splenectomized patients and 88% of non-splenectomized patients had an overall response (defined as a platelet count > 50×10^9/L for 4 wk during the study period) and 38% of splenectomized patients and 61% of non-splenectomized patients had a durable response (platelet count > 50×10^9/L for 6 of the last 8 wk of

the study). In an ongoing long-term extension study of romiplostim that allows dose adjustments to maintain a platelet count between 50-200 × 10^9/L, romiplostim dosage and efficacy have remained stable over 5 years[18,49,50].

Dapsone

Dapsone is a moderate corticosteroid-sparing agent that is usually administered orally at a dose of 75 to 100 mg/d. Dapsone may delay splenectomy for up to 32 mo in patients who have not responded to first-line corticosteroid therapy. However, splenectomized patients have a low response rate[2].

Eltrombopag

Eltrombopag is a nonpeptide small-molecule c-mpl agonist that is taken orally once daily. A recent randomized, placebo-controlled study in patients with ITP refractory to other primary treatments found that eltrombopag was highly effective in raising platelet counts over the 6 mo of the study. Like romiplostim, it was effective in both splenectomized and non-splenectomized patients.

Although eltrombopag has not been studied for as long as romiplostim, data over 3 years indicate that increased platelet counts are maintained without the emergence of drug resistance or cumulative toxicity. Several other drugs in this class are currently in development[51,52].

CONCLUSION

The pathophysiology of ITP is complex and abnormalities of both the B and the T-cell compartments have been identified. The mechanisms of thrombocytopenia involve both increased platelet destruction and, in a significant proportion of cases, impaired platelet production.

Splenectomy has historically been the second-line therapy for adults with ITP in whom achieving a safe platelet count with initial corticosteroid and/or immunoglobulin therapy has failed. Although it still remains the therapeutic modality that offers the highest chance of cure, its position in the therapeutic algorithm of ITP is currently challenged. Rituximab has been shown to have a limited but valuable activity as a splenectomy sparing agent and is generally tolerated very well. The Thrombopoietin-receptor agonists have undergone a formal, systematic investigation and have been licensed for use in adult patients with ITP. These agents appear to be very effective in a high percentage of patients with chronic and refractory disease and appear to have a favorable side-effect profile in the short and medium term. Potential long-term side effects of TPO-receptor agonists remain a concern and suggest their prudent use in young, non-splenectomized patients.

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P- Reviewers: Kim K, Yazdanbakhsh K, Zimmer J
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World J Immunol 2014 March 27; 4(1): I-V
ISSN 2219-2824 (online)

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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.

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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 Immunology
Room 903, Building D, Ocean International Center,
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E-mail: bpgoffice@wjgnet.com
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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

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