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REVIEW

Present and future of immune checkpoint blockade: Monotherapy to adjuvant approaches

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Abstract

Immune regulation of aggressive tumor growth is often outpaced by tumor up-regulation of ligands that inhibit effector immune responses through the activation of immune checkpoints. A few of such checkpoints include programmed death-1 (PD-1), cytotoxic T lymphocyte associated antigen-4 (CTLA-4), lymphocyte activation gene-3, T-cell immunoglobulin and mucin protein-3, Glucocorticoid-induced TNFR family-related receptor (GITR), and killer cell immunoglobulin like receptor. With the exception of GITR, after binding to their respective ligands these checkpoints induce down-modulation of immune responses to prevent autoimmunity. However, such immune mechanisms are co-opted by tumors to allow rapid tumor cell proliferation. Pre-clinical studies in antibody blockade of PD-1 and CTLA-4 have led to promising augmentation of effector immune responses in murine tumor models, and human antibodies against PD-1 and CTLA-4 alone or in combination have demonstrated tumor regression in clinical trials. The development of immune checkpoint blockade as a potential future immunotherapy has led to increasing interest in combining treatment modalities. Combination checkpoint blockade with chemotherapy and radiation therapy has shown synergistic effects in pre-clinical and clinical studies, and combination checkpoint blockade with bacterial vaccine vectors have produced increased effector immune responses in pre-clinical models. The future of immune checkpoint blockade may be as a powerful adjuvant alongside the current standard of care.

Key words: Programmed death-1; Cytotoxic T lymphocyte associated antigen-4; Ipilimumab; Nivolumab; Immune checkpoint

Core tip: Aggressive cancer growth is often characterized by tumor expression of molecules that co-opt effective immune responses through immune checkpoints. Clinical blockade of checkpoints programmed death-1 and cytotoxic T lymphocyte associated antigen-4 and has spurred the discovery of a number of immune checkpoints that may be inhibited in anticancer therapy. The clinical successes of checkpoint blockade have led to increasing interest in combining treatment modalities. Combination checkpoint blockade with chemoradiation has shown synergistic effects, and checkpoint blockade

with bacterial vaccine vectors have produced increased immune responses in pre-clinical models. The future of immune checkpoint blockade may be as a powerful adjuvant alongside the current standard of care.

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INTRODUCTION

Aggressive tumors develop molecular mechanisms to survive in harsh host environments and proliferate in regions of chronic inflammation[1]. Tumor release of factors that promote angiogenesis and disrupt nearby tissue architecture promotes invasion and metastatic spread. The central role of the immune system is to destroy foreign antigens, including those expressed as a result of aberrant cell growth[2]. Proliferating cancer cells may go immunologically unnoticed by the systemic immune response because of tumor cell expression of molecules that inhibit immune effector cells or deplete the tumor microenvironment of key factors for immune cell survival[3,4]. While initial tumor growth is detected and destroyed by robust innate and adaptive immune responses, dormant tumor cells are left behind that are in equilibrium with effector cells of the adaptive immune response[4-7]. Tumor cells will up-regulate ligands and release soluble factors that will inhibit cytotoxic T-lymphocyte activity, and tumor expression of such molecules results in cancer escape from the immune response and proliferation of tumor cells[3,4]. This process of immune-mediated editing of tumor cells, equilibrium of tumor growth maintained by antigen-specific immune responses, and ultimate escape of the tumor from immune surveillance is termed the "Three E's" of cancer immunoediting[3,7]. Recent advances in immunotherapy have sought to intervene in this process of tumor immune escape[8].

Immune checkpoints are cell surface receptors expressed on a variety of immune cells that, under normal circumstances, prevent peripheral autoimmunity during inflammatory responses[9]. Poorly immunogenic cancer cells can express the ligands of such immune checkpoints, resulting in immune effector inhibition in the tumor microenvironment. Blockade of such immune checkpoints attempts to block the inhibitory interaction between tumor cells and T-cells and promote tumor-specific T-cell activation. This review will discuss the cellular pathways of immune checkpoints, pre-clinical and clinical studies in checkpoint blockade, and the potential future of immune checkpoint inhibition as an adjuvant to chemotherapy, radiation therapy, and bacterial vaccine vectors.

IMMUNE CHECKPOINTS: CELLULAR AND *IN VIVO* MECHANISMS

While immune checkpoints are known to down-regulate immune effector cells, studies suggest that each checkpoint induces immune inactivation through discrete cellular mechanisms[10-13]. Immune checkpoint receptor-ligand interactions are not limited to T-lymphocytes and tumors, however. Checkpoint receptors are expressed on a variety of immune cells, including natural killer (NK) cells and cells derived from the myeloid lineage. Checkpoint signaling in these cells is implicated in infection, prevention of autoimmunity, and tumor immune evasion. The following sections will focus on immune checkpoints in T-lymphocytes and their involvement in tumor-lymphocyte interactions. Checkpoints programmed death-1 (PD-1), cytotoxic T-lymphocyte antigen-4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), and T-cell immunoglobulin and mucin protein-3 (TIM-3) are a few of a number of down-regulators of T-cell function.

PD-1

PD-1 is a transmembrane protein that transmits inhibitory signals upon engagement with its ligands programmed death ligand-1 and -2 (PD-L1, PD-L2) and is responsible for down-regulating T-cell activation[3,11,12,14]. PD-1 is largely expressed in activated T-cells in the periphery and prevents destruction of host tissues expressing PD-L1 during inflammatory responses[15]. In addition to host epithelial cells, PD-L1 is constitutively expressed in myeloid cells, professional antigen presenting cells (APCs), lymphoid cells, and cancer cells, while PD-L2 expression is inducible and largely limited to APCs[12,16,17]. Notably, PD-L2 also binds to repulsive guidance molecule b, and this interaction is responsible for respiratory tolerance[18]. Cell signaling through PD-1 plays a significant role in preventing autoimmunity, as evidenced by the development of lupus-like glomerulonephritis and arthritis, cardiomyopathy, as well as increased levels of IgG2b, IgG3 and IgA in PD-1$^{-/-}$ knockout mice[19-21]. The structure of PD-1 includes the cytoplasmic structural motifs immunoreceptor tyrosine-based inhibitor motif and immunoreceptor tyrosine-based switch motif (ITSM); upon ligand binding to PD-1, ITSM recruits phosphatases SHP-1 and SHP-2 which are capable of inhibiting downstream kinases that in turn inhibit T-cell proliferation, cytokine release, and cytotoxic function[4-7,22] (Figure 1). In addition, PD-1 ligation results in mitigated phosphorylation of the ZAP70/CD3ζ signalosome, resulting in downregulation of the T-cell receptor signaling pathway and subsequent T-cell activation[1,4,23]. Finally, PD-1 has been shown to downmodulate IFN-γ production as evidenced by relatively increased IFN-γ levels after PD-1/PD-L blockade[2-4,20]. IFN-γ is a marker of the Th1 phenotype

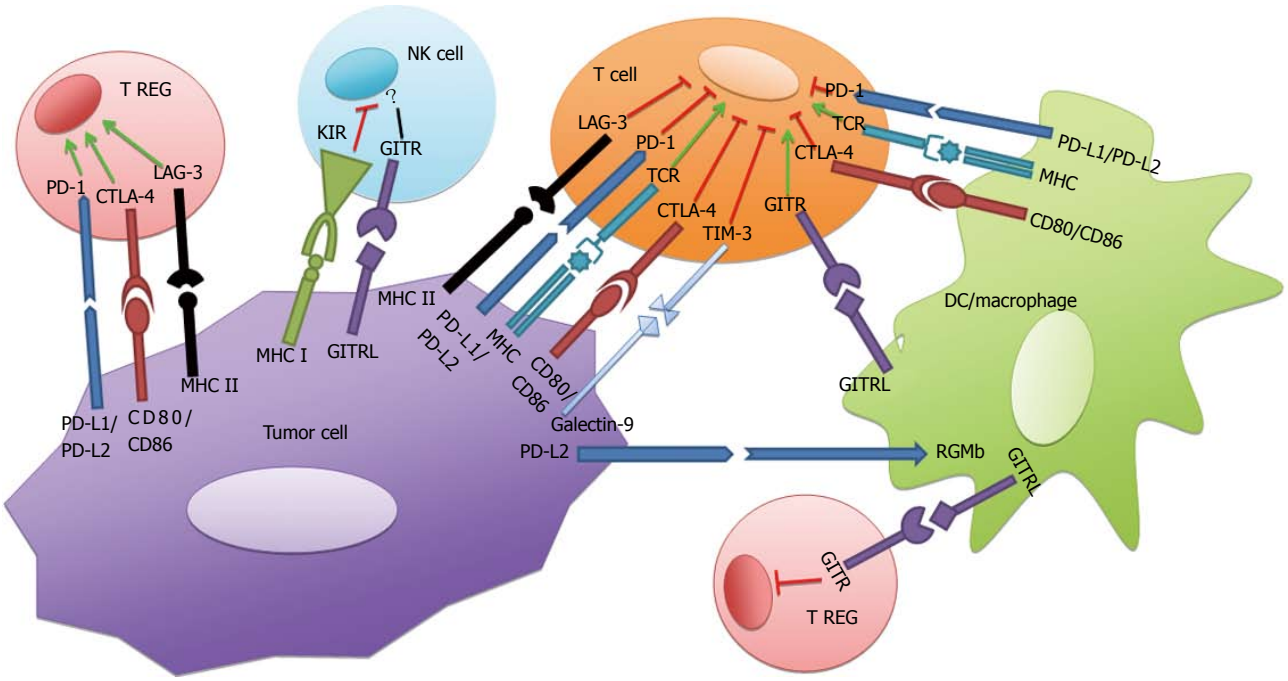

Figure 1 Stimulatory and inhibitory responses mediated by immune checkpoints. Depicted are interactions between immune checkpoints and their ligands as well as their cellular effects. Stimulatory effects are depicted with green arrows, and inhibitory effects are depicted with red symbols. The effect of GITR ligation in NK cells is not yet fully understood. T REG: Regulatory T cell; DC: Dendritic cell; NK: Natural killer; MHC: Major histocompatibility complex; PD-1: Programmed death-1; KIR: Killer cell immunoglobulin like receptor; TCR: T-cell receptor; LAG-3: Lymphocyte activation gene-3; TIM-3: T-cell immunoglobulin and mucin protein-3; CTLA-4: Cytotoxic T lymphocyte associated antigen-4.

as well the activation of effector CD8+ T-cells, and decreased IFN-γ production may reflect a depressed immune response.

CTLA-4

Similar to PD-1, CTLA-4 downregulates T-cell effector function, although the action of CTLA-4 is manifested largely in naïve, resting T-cells and occurs by a pathway distinct from PD-1[3,4,24]. CTLA-4 is a co-inhibitory receptor, competing with the co-stimulatory receptor CD28 for their shared ligands B7-1 (CD80) and B7-2 (CD86)[4-7,25] (Figure 1). By sequestering CD80/CD86, CTLA-4 is capable of inhibiting T-cell receptor (TCR) function while preventing CD28 from binding to its ligands and promoting TCR-mediated T-cell activation. Importantly, CTLA-4 blockade was the first of the immune checkpoints to exhibit anti-tumor immunity *in vivo*, which provided evidence for the activity of CTLA-4 in promoting tumorigenesis through cancer immune escape[3,5-7,26,27]. The purpose of CTLA-4 is to check T-cell activation by attenuating T-helper cell activity and up-regulating the immunosuppressive activity of Tregs. Tregs are CD4+ lymphocytes that down-regulate immune effector function, typically to prevent autoimmunity. In the tumor microenvironment, cancer cells may up-regulate Treg activity through ligand-receptor interactions, leading to the inactivation of effector T-cell responses. The critical role of CTLA-4 in T-cell homeostasis is demonstrated by significant lymphocyte-mediated multi-organ destruction in CTLA-4$^{-/-}$ knockout mice, of a magnitude more severe than in PD-1 deficient mice and occurring earlier in life[3,9,24,28,29]. While the signaling pathway by which CTLA-4 inhibits TCR is still unclear, studies show that the cytoplasmic domain of CTLA-4 activates SHP-2 and phosphatase PP2A, resulting in inhibition of kinases ZAP-70, LCK, and FYN that are specific to the TCR and mediate T-cell activation[9-13,30,31].

LAG-3

Responsible for down-regulating effector T-cell responses by promoting Treg-mediated immune suppression, LAG-3 is a CD4 receptor homologue of the Ig superfamily expressed in activated NK and T-cells (Figure 1)[3,10-14,32]. Like CD4, LAG-3 interacts with the major histocompatibility complex class II (MHCII) but is not responsible for MHCII-restriction in CD4-independent lymphocytes[3,11,12,14,15,33]. Instead, LAG-3 regulates antigen-specific lymphocyte responses through its activity in CD4+CD25+ regulatory T cells[12,15-17,34]. Increased lymphocytic expansion was observed in LAG-3 deficient T-cells stimulated with staphylococcal enterotoxin B (SEB), and LAG-3 deficient T-cells produced higher levels of IFN-γ and IL-2 after SEB stimulation[12,16,17,19-21,35]. As a result of its involvement in Treg function, LAG-3 promotes self-antigen tolerance and prevents autoimmunity. This is evidenced by the finding that LAG-3-deficient non-obese diabetic (NOD) mice develop an accelerated insulitis with extensive CD4+ and CD8+ pancreatic infiltration, suggested a role for LAG-3 in the regulation of activated CD4+ and CD8+ lymphocyte responses[19-21,32,36,37]. Unlike in PD-1

and CTLA-4 deficient mice, LAG-3 deficiency does not produce severe or obvious autoimmunity, and while the LAG-3 signaling pathway is still unclear, it does not appear that there is major overlap between the LAG-3 and PD-1 pathways[4-7,22,38]. Importantly, LAG-3 blockade combined with vaccines has shown increased antitumor immunity mediated by activated CD8+ T-cells. LAG-3 inhibition of influenza hemagglutinin (HA)-specific CD8+ T-cells combined with recombinant vaccinia virus expressing wild-type HA protein in a mouse model expressing influenza HA as self antigen and modified as tumor antigen resulted in increased intratumoral CD8+ activation as well as increased levels of antigen-specific CD8+ cells as well as tumor parenchyma destruction[39]. Such evidence suggests a role for LAG-3 blockade in future anticancer therapies[40].

TIM-3

T-cell immunoglobulin and mucin protein-3 down-regulates Th1 immune responses in the periphery and prevents autoimmunity after binding to its ligand galectin-9 (Figure 1)[41]. In addition to certain tumors, TIM-3 is expressed on T-cells and nerve cells[42]. Binding of TIM-3 to its ligand induces phosphorylation of a tyrosine residue on the cytoplasmic tail of TIM-3 by interleukin inducible T cell kinase, resulting in downstream inhibition of Th1 effector function[43]. TIM-3 blockade *in vivo* has resulted in autoimmune disease including encephalomyelitis as well as increased activation of macrophages, possibly resulting from released inhibition on Th1 mediated immune responses[44]. Similar to LAG-3, TIM-3 promotes self-antigen tolerance demonstrated by the observation that TIM-3 blockade results in accelerated diabetes in NOD mice, possibly by reducing Treg activity in attenuating the Th1 immune response[45]. TIM-3 blockade has also shown promise as a potential anticancer therapy, as it results in an increased ratio of CD8+ to CD4+ tumor infiltrating lymphocytes in a murine colon cancer model, especially in cases of tumor regression, as well as increased IFN-γ production by T-cells; similar results and tumor suppression are observed after anti-TIM-3 therapy in murine BALB/c colon adenocarcinoma and fibrosarcoma models[46]. Additional studies are needed to clearly define the TIM-3 signaling pathway and its immunomodulatory effects.

Glucocorticoid-induced TNFR family-related receptor

Unlike the immune checkpoints mentioned thus far, Glucocorticoid-induced TNFR family-related receptor (GITR) is a co-stimulatory immune checkpoint that is expressed on CD4+ and CD8+ T-lymphocytes as well as Tregs, NK cells, dendritic cells (DC) and monocytes, and binds to its ligand expressed on APCs and endothelial cells (Figure 1)[47-49]. While GITR is expressed at a low-level on resting CD4+ and CD8+ T-cells, it is temporarily upregulated 24-72 h after an immune stimulus; conversely, GITR is constitutively expressed on Tregs[48,49]. GITR ligation results in an augmented effector T-cell response and resistance to activation induced cell death as well as to the suppressive effects of Tregs[50-52]. The ligation of GITR by agonist antibody DTA-1 has shown to cause Treg lineage instability through loss of FoxP3 and results in increased cytotoxic T-lymphocyte to Treg ratios[51]. Ligation of GITR, whether *via* an agonist antibody or GITRL-expressing DC vaccine, has resulted in murine melanoma regression and increased intratumoral effector T-cell to Treg ratios as a consequence of impairment of Treg infiltration into the tumor microenvironment and instability caused by loss of FoxP3[51]. The immune activating effects of GITR ligation are dependent on GITR expression in both effector T-cells as well as Tregs[51]. Preclinical studies of GITR in combination with other immunotherapies have demonstrated promising antitumor effects. In fibrosarcoma-bearing mice, co-administration of anti-CTLA-4 and anti-GITR antibodies led to synergistic tumor eradication mediated by tumor-specific CD4+ and CD8+ T-cells[53]. Similarly, combination adoptive T-cell therapy and anti-GITR antibody administration results in increased effector responses as measured by IFN-γ, TNFα and CD107a in a murine fibrosarcoma model[54]. While the precise mechanism of GITR ligation and its downstream consequences have yet to be defined, GITR modulation through agonist antibodies or GITRL expressing DC vaccines may be an efficacious avenue in immune checkpoint antitumor therapy.

Killer cell immunoglobulin like receptor

Killer cell immunoglobulin like receptor (KIR) is an immune checkpoint that primarily regulates NK cell activation (Figure 1). Cytolytic $CD56^{dim}CD16+$ NK cells express KIR which is specific for the HLA class I allele, and tumor infiltrating NK cells expressing KIR have been found to be downregulated by tumor-KIR interactions[55,56]. The precise intracellular mechanisms of KIR are as yet unclear, but KIR genes and their respective HLA class I ligands have been identified in non-small cell lung cancer (NSCLC) and kidney cancer patients, providing a potential role for NK cells in antitumor immune responses[57]. One study found that a high percentage of NSCLC patients had KIR-expressing NK cells compared to controls (P = 0.0004), and those NK cells were dysfunctional in terms of effector cytokine expression and overall cytotoxicity[58]. The involvement of KIR in tumor mediated immune suppression has led to the development of KIR-blocking human monoclonal antibody Lirilumab, which is currently being tested in combination with human anti-PD-1 and anti-CTLA-4 antibodies (NCT01750580, NCT01714739). Discovery of additional immune checkpoints on cells outside of the T-cell subset has led to increasing opportunities in overcoming tumor mediated immune suppression by blocking tumor-cell interactions involving a variety of immune cells. NK cells may become the next target for immune checkpoint blockade.

Pre-clinical studies: PD-1, PD-L1 and CTLA-4

The first steps to understanding the role of PD-1 and its ligands in tumor survival was the characterization of PD-L1 expression almost exclusively on cancer cells[59]. Dong and colleagues showed that PD-L1 expression on tumor cell lines resulted in increased apoptosis of antigen-specific T-cells *in vitro* and *in vivo*, and that the apoptotic effect was mediated by multiple T-cell receptors including PD-1[59]. Early studies showed that tumor PD-L1 expression rendered tumor cells less susceptible to cytotoxic T-lymphocyte activity, and the interruption of the interaction between PD-1 and PD-L1 could re-establish tumor susceptibility to immune mediated cytotoxicity[60]. This discovery established the potential for PD-1 or PD-L1 blockade as a means toward increased antitumor efficacy by cancer therapeutics. Iwai and colleagues further confirmed the dependence of some aggressive and poorly immunogenic tumors on immune checkpoint pathways to evade immune responses. Their group transgenically expressed PD-L1 in a murine myeloma cell line and demonstrated decreased cell lysis *in vitro* by cytotoxic T-cells compared to tumor cells that did not express PD-L1[60]. When grown in syngeneic mice and treated with anti-PD-L1 antibody, murine myeloma cell lines expressing PD-L1 had transiently diminished growth; the same tumors did not grow in PD-1 deficient mice[60]. Blank *et al*[61] further characterized tumor resistance to CD8+ T-cell effector function by exposing PD-L1-expressing cancer cell lines to PD-1-deficient effector T-cells. Compared to wild-type, PD-1 deficient lymphocytes had increased proliferative, cytotoxic, and cytokine-producing activity, and this phenomenon is reproducible *in vivo* with anti-PD-L1 antibody in the effector phase[61]. This finding illustrated that if PD-1 expressed by lymphocytes could be blocked such that it could no longer interact with its ligand, and if this state essentially mimicked PD-1 deficiency as in the study by Blank *et al*[61], then future therapeutics could reverse immune inhibition in the tumor microenvironment and allow the host immune response to eliminate tumor. Confirming this concept, Hirano and colleagues demonstrated that antibody blockade of either PD-1 or PD-L1 interrupts tumor immune evasion by reversing resistance to lymphocyte effector function[62]. Together, these studies provided pre-clinical evidence that the PD-1/PD-L1 axis is crucial to tumor evasion of the immune response and blockade of this interaction is an important opportunity for suppressing tumor growth *in vivo*.

Food and Drug Administration approval for CTLA-4 occurred as preclinical studies were beginning to show that PD-1 blockade could be another opportunity for anti-cancer immunotherapy. The discovery that PD-1 and CTLA-4 signal through distinct and synergistic pathways naturally led to testing both in combination checkpoint blockade in preclinical tumor models[20,23]. In a murine B16 melanoma model, Curran and colleagues showed that combination anti-CTLA-4 and anti-PD-1 therapy with concurrent Flt3-ligand (Fvax) vaccine resulted in a 50% tumor rejection rate, compared to 25% with anti-PD-1 and 10% with anti-CTLA-4 each with Fvax[63]. Notably, combination blockade increased the ratio of effector T-cells to regulatory T-cells and myeloid derived stem cells (MDSCs) in the tumor microenvironment, resulting in greater local T-cell activation and anti-tumor inflammation. This study provided a mechanism for the efficacy of combination therapy centering on the importance of the effector T-cell response and down-regulation of inhibitory cell lines like Tregs and MDSCs in tumor elimination. Duraiswamy *et al*[64] elucidated the mechanism for increased effector T-cell activity and tumor rejection after anti-PD-1 and anti-CTLA-4 combination blockade in a murine colon cancer model[64]. Lymphocytes that express both PD-1 and CTLA-4 exhibited cellular dysfunction in their inability to produce cytokines or proliferate, while simultaneous antibody blockade of both co-receptors reversed T-cell dysfunction including inhibition of Treg function[64]. Similarly, in a murine glioblastoma (GBM) model, Wainwright *et al*[65] found that combination inhibition of indoleamine 2,3-dioxygenase, CTLA-4 and PD-1 resulted in decreased intratumoral Tregs with increased T-cell activation and long-term survival in tumor-bearing mice. Together, these studies emphasize the greater utility of PD-1 and CTLA-4 combination blockade relative to single checkpoint blockade in cancer immunotherapy, and they show that tumor regression occurs *via* effector lymphocyte activation. Inhibition of both T-cell co-receptors fully allows activation and proliferation of antigen-specific anti-tumor T-cells and prevents Treg suppression, resulting in significant suppression of tumor growth in animal models.

CLINICAL STUDIES IN PD-1 AND CTLA-4 BLOCKADE

Anti-CTLA-4 therapy

The first immune checkpoint inhibitor to be FDA approved for clinical use, anti-CTLA-4 antibody, or Ipilimumab, has been tested in a variety of cancer types with an emphasis on melanoma therapy. One of the earliest clinical trials testing anti-CTLA-4 therapy was done in metastatic melanoma, in which 14 patients were dosed with 3 mg/kg of Ipilimumab alongside gp100 vaccine and 21% experienced objective response, with two patients having complete regression[66]. This study also demonstrated the serious adverse autoimmune events associated with anti-CTLA-4 therapy, as 43% of patients experienced grade 3 or 4 adverse effects including enterocolitis and hypophysitis. Ipilimumab therapy for advanced metastatic melanoma continued to be tested in phase 2 and 3 clinical trials with encouraging tumor regression, but immune-related adverse events persisted in each study. In their phase II dose ranging study of 217 participants, Wolchok *et al*[67] established that the best

Table 1 Clinical study outcomes of ipilimumab (anti-cytotoxic T lymphocyte associated antigen-4) in solid tumors

Ref.	Phase	Cancer type	No. of patients	Dose (mg/kg)	Outcome
McDermott *et al*[73]	3	Melanoma	676	3	3-yr survival rate 25%
Hodi *et al*[74]	3	Melanoma	676	3	BORR: 10.9%
Robert *et al*[70]	3	Melanoma	676	3	BORR: 37.5%
Margolin *et al*[71]	2	Melanoma with brain metastasis	93	10	[1]Cohort A: 18% disease control [2]Cohort B: 5% disease control
Di Giacomo *et al*[69]	N/A	Melanoma	27	10	2-yr survival rate 23.5%
Yang *et al*[78]	2	Pancreatic adenocarcinoma	27	3	No responders
O'Day *et al*[68]	2	Melanoma	155	10	BORR: 5.8%
Wolchok *et al*[67]	2	Melanoma	217	10	BORR: 11.1%
Yang *et al*[78]	2	RCC	40	3	PR: 12.5%
Phan *et al*[66]	1	Melanoma	14	3	OR: 21%

[1]Cohort A includes neurologically asymptomatic patients not receiving corticosteroids; [2]Cohort B includes symptomatic patients receiving corticosteroids. Dose indicates dose of Ipilimumab. Outcomes are best outcomes in each study, in patients receiving Ipilimumab only. BORR: Best overall response rate; RCC: Renal cell carcinoma; PR: Partial response; OR: Objective response; N/A: Not available.

overall response rate (BORR) of 11.1% was achieved with 10 mg/kg Ipilimumab, as compared to 4.2% with 3 mg/kg and 0% for 0.3 mg/kg. O'Day *et al*[68] applied the 10 mg/kg dosing scheme in pretreated advanced melanoma, and they observed a BORR of 5.8%, a disease control rate of 27%, and a median improved overall survival (OS) of 10.2 mo; 22.3% experienced immune related adverse events in this cohort[68]. Even in heavily pretreated melanoma, treatment with 10 mg/kg Ipilimumab resulted in promising disease control rates at 24 and 60 wk of 29.6% and 15%, respectively, with a one year survival rate of 34.8%, providing evidence that immune checkpoint inhibitors could still be used as a last resort in treatment resistant disease[69]. Robert *et al*[70] corroborated these results and found that even in pre-treated melanoma with disease progression, 75% treated with Ipilimumab alone experienced disease regression[70]. Brain metastasis did not preclude individuals from disease control with Ipilimumab, and in one study 18% of those neurologically asymptomatic from intracranial metastasis experienced overall disease control[71]. Importantly, Ipilimumab-induced disease regression in melanoma was durable, as an analysis of three phase II trials demonstrated a four year survival rate of 19.7%-28.4% at a dose of 10 mg/kg[72]. In a recent phase III trial of 676 individuals with metastatic melanoma treated with Ipilimumab alone, 25% survived at two and three years and only 8% experienced drug related toxicity[73,74]. The aforementioned clinical studies are summarized in Table 1.

As a result of encouraging clinical trial outcomes, a number of studies have ensued attempting to define markers of response to Ipilimumab therapy. Hamid and colleagues found that there is a significant association between clinical activity of Ipilimumab and indoleamine 2,3-dioxygenase levels (P = 0.012), FoxP3 expression (P = 0.014), as well as increased tumor infiltrating lymphocytes at three weeks after initiating therapy (P = 0.005)[75]. Moreover, there is a significant increase in expression of immune-related genes such as immunoglobulins, granzyme B, perforin-1, granulysin, and CD8 beta subunit and a decrease in expression of melanoma and cancer genes after therapy with Ipilimumab[75]. Moreover, Weber *et al*[76] in a study of humoral responses after Ipilimumab found that serologic activity against antigen NY-ESO-1 increased up to five fold at 12 wk in 10%-33% of individuals, and there were increased antibody levels against antigens p53, SSX2, MAGE-A4 and Melan-A[76]. Furthermore, they found significantly increased activated CD4 and CD8 and memory CD4 cells but not FoxP3 Treg or CD8 cells after 4 wk of Ipilimumab therapy[76]. While some had speculated that HLA status may affect response to anti-CTLA-4 therapy, Wolchok *et al*[77] found that HLA status had no association with overall survival or adverse events.

Antitumor responses with Ipilimumab are not limited to melanoma therapy; clinical responses have been observed in renal cell carcinoma (RCC), non-Hodgkin lymphoma, and prostate cancer, but such responses were not observable in pancreatic ductal adenocarcinoma[78-82]. Overall, Ipilimumab has demonstrated that immune checkpoint blockade alone can produce modest tumor regression in a number of solid tumors with some grade 3 and 4 immune related adverse events. As a result, a number of groups have begun to test other checkpoint molecules that could be blocked clinically to have a greater impact on antitumor immunity with similar or more limited side effect profiles.

Anti-PD-1 therapy

Anti-PD-1 antibodies Nivolumab and Lambrolizumab have begun to show promising results in melanoma clinical trials. In their phase I trial of anti-PD-1 antibody at a dose of 0.1-10 mg/kg in 296 patients, Topalian and colleagues reported cumulative response rates of 28% with melanoma, 27% with RCC, and 18% with NSCLC[83]. Responses were durable with 64.5% responding at one year[83]. Notably, 36% with PD-L1 positive cancers also had an objective response (P = 0.006), although those with PD-L1 negative tumor were

Table 2 Clinical study outcomes of anti-programmed death-1 or anti-programmed death ligand-1 antibody in solid tumors

Ref.	Phase	Cancer type	No. of patients	Antibody type	Outcome
Hamid *et al*[75]	2	Melanoma	135	Lambrolizumab	RECIST: 1.1; RR: 38% (95%CI: 25-44)
Weber *et al*[85]	1	Melanoma	90	Nivolumab	RECIST: 1.1; RR: 25%
Brahmer *et al*[86]	1	Advanced cancers	207	Anti-PD-L1	OR: 6%-17%
Topalian *et al*[83]	1	Advanced cancers	296	Nivolumab	OR: 18%-36%

Dose indicates dose of anti-PD-1 antibody. RECIST: Response evaluation criteria in solid tumors; OR: Objective response, as defined by partial or complete tumor regression; RR: Response rate; PD-L1: Programmed death ligand-1.

not precluded from responding[83]. In a follow-up study, Lipson *et al*[84] confirmed the durability of response to anti-PD-1 therapy by reporting that in patients who experienced objective responses, all continued to have a complete response after three years, except for one patient with melanoma who had a partial response that was stable for 16 mo off therapy, and whose recurrent disease was successfully treated with anti-PD-1 re-induction therapy. In a phase I study of advanced pretreated melanoma, Weber *et al*[85] observed a 25% RECIST 1.1 response rate and found that increased peripheral Tregs and decreased antigen specific T-cell were associated with progression, while PD-L1 staining was associated with a response to Nivolumab therapy, but not all who responded stained PD-L1 positive. Moreover, high pre-treatment levels of MART-1 and NY-ESO-1 specific CD8+ T-lymphocytes were associated with progression. In a study of Lambrolizumab in advanced melanoma, Hamid and colleagues observed an overall response rate of 38% with the highest response being 52% at a dose of 10 mg/kg[75]. Finally, there has been some investigation into blockade of the PD-1 ligand, PD-L1, as a more effective anti-tumor therapy. A phase I study in 207 patients with an array of advanced solid cancers treated with anti-PD-L1 antibody observed an objective response in 17.3% with melanoma, 11.7% with RCC, 10.2% with NSCLC and 6% with ovarian cancer[86]. Expression of PD-L1 on the tumor cell surface seemed to correlate with response to anti-PD-L1 therapy. These clinical studies are summarized in Table 2.

Combination checkpoint blockade

Checkpoint blockade monotherapy with anti-PD-1 and anti-CTLA-4 antibodies have shown durable, though modest, tumor regression, but with the understanding that the two checkpoints function through discrete intracellular pathways, their clinical use together in double checkpoint blockade appeared to be a potentially additive or even synergistic therapy. Wolchok and colleagues produced encouraging results in their phase I trial of advanced melanoma treated with 1 mg/kg Nivolumab and 3 mg/kg Ipilimumab concurrently[87]. At maximum tolerated doses, 53% of patients had an objective response with an overall tumor regression of greater than 80% for all patients. Importantly, the side effect profile of combination therapy was similar to monotherapy and was largely reversible, indicating that patients may be treated and any side effects managed on an outpatient basis with corticosteroids. As a consequence of such promising clinical results, a number of clinical trials studying Nivolumab and Ipilumab in combination are currently ongoing (Table 3). These include studies of advanced stage and untreated melanoma, renal cell carcinoma, metastatic colon carcinoma, and recurrent glioblastoma involving dose escalation as well as sequential versus concurrent therapy. Whether combination checkpoint blockade extends to other solid tumors is yet to be seen, but this period certainly marks the advent of combination checkpoint blockade as a potential future anti-cancer therapy. It is important to note that there may be significant adverse events associated with immune checkpoint blockade, including enterocolitis and hypophysitis, among other immune related adverse events[67].

FUTURE THERAPIES

Immunotherapy with checkpoint blockade provides an opportunity for future adjuvant therapy with other treatment modalities, including.

Checkpoint blockade and chemotherapy

The emergence of checkpoint blockade as a putative therapy for malignant neoplasms has raised the possibility of combination cytotoxic and immunotherapeutic regimens as avenues for tumor eradication (Table 4). Chronic pro-cancer inflammation mediated by cell-intrinsic somatic mutations and cell-extrinsic pathways such as vascular proliferation has historically prevented total tumor eradication by chemotherapy[1]. Immune checkpoint blockade may reprogram the inflammatory tumor microenvironment by harnessing active tumor-specific effector and memory CD4+ and CD8+ T-cells and thereby shifting tumor-associated inflammation from a pro-cancer to an anticancer state[1]. A number of pre-clinical and clinical studies have combined various cytotoxic therapies with checkpoint inhibitors with promising results.

Anti-CTLA-4 antibody combined with chemotherapy in murine models of lung cancer has demonstrated synergistic effects. Lesterhuis *et al*[88] found that 60% of mice receiving anti-CTLA-4 antibody and

Table 3 Ongoing clinical Trials in combination anti-programmed death-1 and anti-cytotoxic T lymphocyte associated antigen-4 Blockade

Identifier	Phase	Cancer type	Checkpoint antibodies	Status
NCT02060188	2	Microsatellite High (MSI-H) Colon Cancer	Nivolumab, ipilimumab	Recruiting
NCT01454102	1	NSCLC	Nivolumab, ipilimumab	Recruiting
NCT01472081	1	RCC	Nivolumab, ipilimumab	Recruiting
NCT01024231	1b	Melanoma	MDX-1106, ipilimumab	Active
NCT01928394	1/2	TNBC, GC, PC, SCLC, BC	Nivolumab, ipilimumab	Recruiting
NCT02017717	2b	GBM	Nivolumab, ipilimumab	Recruiting
NCT01927419	2	Melanoma	Nivolumab, ipilimumab	Active
NCT01844505	3	Melanoma	Nivolumab, ipilimumab	Active
NCT01592370	1	Hematologic malignancy	Nivolumab, ipilimumab	Recruiting
NCT01783938	2	Melanoma	Nivolumab[1], ipilimumab	Recruiting

[1]Nivolumab is sequentially dosed with Ipilimumab in this study, whereas in all other studies it is concurrently dosed. Identifier refers to Clinicaltrials.gov identifier. NSCLC: Non-small cell lung cancer; RCC: Renal cell carcinoma; TNBC: Triple negative breast cancer; GC: Gastric cancer; PC: Pancreatic cancer; SCLC: Small cell lung cancer; BC: Bladder cancer; GBM: Glioblastoma.

Table 4 Clinical study outcomes of combination chemotherapy and immune checkpoint blockade

Ref.	Phase	Cancer type	Treatment combination	Outcome
Robert *et al*[70]	3	Melanoma	Ipilimumab + dacarbazine	3-yr survival 20.8% ($P < 0.001$)
Lynch *et al*[94]	2	NSCLC	Ipilimumab + paclitaxel, carboplatin (phased)	irPFS HR: 0.72, $P = 0.05$
Reck *et al*[95]	2	SCLC	Ipilimumab + paclitaxel, carboplatin (phased)	irPFS HR: 0.64, $P = 0.03$
Hersh *et al*[90]	2	Melanoma	Ipilimumab + dacarbazine	ORR: 14.3% (95%CI: 4.8-30.3)

All outcomes are in combination therapy treatment arms. NSCLC: Non-small cell lung cancer; SCLC: Small cell lung cancer; irPFS: Immune-related progression free survival; HR: hazard ratio; ORR: Objective response rate.

gemcitabine had complete tumor regression, compared to 13% in those receiving anti-CTLA-4 alone and 8% in those receiving gemcitabine alone. Importantly, this combination effect was timing dependent, with greatest tumor regression observed in mice receiving concomitant anti-CTLA-4 and gemcitabine. In a murine mesothelioma model, anti-CTLA-4 antibody administered between cisplatin dosing intervals inhibited tumor regrowth and prolonged survival in mice receiving anti-CTLA-4 plus cisplatin therapy compared to cisplatin alone (38 d *vs* 30 d, $P = 0.0139$)[89]. Moreover, combination anti-CTLA-4 plus cisplatin therapy produced increased intratumoral CD4+ and CD8+ lymphocytic infiltrate and increased expression of markers of lymphocyte activation including interleukin-2 (IL-2), IFN-γ, granzyme B and perforin[89].

In line with pre-clinical findings, clinical studies in metastatic melanoma have shown improved disease control with combination Ipilimumab and chemotherapy. In a phase II study of Ipilimumab and dacarbazine in metastatic melanoma, those who received combination therapy had an objective response rate (ORR) of 14.3% *vs* 5.4% in those who received dacarbazine plus placebo, and a median OS of 14.3 mo *vs* 11.4 mo[90]. Those who received combination therapy in this study did experience moderately increased immune mediated adverse events compared to the placebo arm (65% *vs* 53.8%)[90]. A phase III study of untreated metastatic melanoma had similar findings of OS in those receiving Ipilimumab plus dacarbazine (11.2 mo *vs* 9.1 mo) with durable improvements in survival rates at three years (20.8% *vs* 12.2%, $P < 0.001$). Combination checkpoint blockade plus chemotherapy has even shown disease control in advanced melanoma with brain metastasis, with Ipilimumab plus fontemustine producing disease control in 50% with intracranial disease and in 46.5% with stage IV disease without brain metastasis[91]. Retrospective studies have shown that even regional chemotherapy in melanoma followed by Ipilimumab has produced higher complete rates than IL-2 alone (33% *vs* 0%, $P = 0.021$)[92]. Importantly, combination anti-CTLA-4 in melanoma not only improves tumor regression, but Ipilimumab plus dacarbazine has demonstrated increasing quality-adjusted survival over time compared to dacarbazine alone, with a difference of 3.28 mo at 4 years ($P = 0.0074$)[93].

The benefits of Ipilimumab combined with chemotherapy are not limited to melanoma, but have been observed in NSCLC and small cell lung cancer (SCLC) as well. In a phase II trial of stage IIIB/IV NSCLC treated with Ipilimumab plus paclitaxel and carboplatin, phased Ipilimumab with chemotherapy improved immune-related progression free survival (irPFS) compared to concurrent Ipilimumab and control treatments (5.7, 5.5, 4.6 mo, respectively), as well as PFS (5.1, 4.1, 4.2 mo, respectively) and median OS (12.2, 9.7, 8.3 mo, respectively)[94]. Similarly, in a phase II study of SCLC treated with Iplimumab plus paclitaxel and carboplatin, only phased Ipilimumab improved irPFS compared to the control [hazard ratio (HR) =

0.64, $P = 0.03$], but without any improvement in PFS or OS[95].

While a number of clinical studies show a potential adjunctive role for anti-CTLA-4 therapy with chemotherapy, combination anti-PD-1 antibody with cytotoxic regimens are just entering pre-clinical and clinical studies. Hasan *et al*[96] demonstrated that doxorubicin down-regulates surface PD-L1 expression in breast cancer, leading to anti-apoptotic effects through intracellular regulation by PD-L1 of apoptotic machinery[96]. Their findings emphasize the need to further elucidate the cell-level interactions between chemotherapy, PD-1 and PD-L1 in cancers being targeted by anti-PD-1 therapy. Moreover, case reports have shown severe hypersensitivity reactions including autoimmune demyelinating polyneuropathy in those who received anti-PD-1 therapy followed by vemurafenib[97]. The mechanism behind such adverse events is unclear, but it is evident that the safety of combining anti-PD-1 with cytotoxic therapies must be established. Currently, ongoing phase I trial Checkmate 012 (NCT01454102) is recruiting those with stage IIIB/IV NSCLC for treatment with Nivolumab in combination with a number of chemotherapeutic regimes.

Given what studies have shown regarding combination Ipilimumab and chemotherapy, it is worthwhile pursuing such combination regimens with other checkpoint inhibitors in pre-clinical and clinical settings. It is important to maintain that cytotoxic therapy is successful only if the host is able to mount an appropriate immune response to counter procancer inflammation and immune resistance of cancer cells; in those whose cancers are particularly aggressive because of up-regulation of immune checkpoint ligands, checkpoint inhibitor therapy provides the opportunity to combat tumor regrowth and allow cytotoxic therapy to fully eradicate tumor[98].

Checkpoint blockade and radiation therapy

Radiation therapy produces effects locally and systemically to promote tumor regression. Locally, tumor cell destruction through apoptotic pathways is closely interlinked with increased inflammatory changes that recruit antitumor immune responses. Ionizing radiation induces DNA damage that initiates a cascade of intracellular and extracellular events. Intracellularly, caspases inactivate proteins responsible for DNA damage recognition and repair. Extracellularly, a number of "stress signals" are expressed that attract immune-mediated cell death[99,100]. The expressed stress signals along with tumor antigens released by damaged tissue not only promote direct tumor destruction through cell mediated immunity, but provide an immunogenic substrate for an increased inflammatory response[2,101-107]. Early studies showed that T-cells were involved in tumor regression in irradiated mice, and it is now understood that radiation therapy modulates the tumor microenvironment through increased cytokine expression and antigen expression[108-111].

In effect, radiation therapy recreates an immunogenic tumor microenvironment in the place of one that was immunosuppressive.

Systemically, radiation therapy may promote tumor regression in distant sites through the abscopal effect. The abscopal effect is an immune-mediated phenomenon, involving dendritic cell presentation of dying tumor cell antigens to antigen-specific T-cells, resulting in activation of anti-tumor T-cells that may destroy tumor at peripheral sites[112]. Such a systemic effect of local radiation therapy has been observed in mammary carcinoma in mice and human hepatic carcinoma after radiation of bony metastases as well as adenocarcinoma[113-115]. Such observations provide promise for future combination therapies involving immunotherapy and local radiation therapy to destroy even metastatic disease.

The non-redundancy of immune checkpoint signaling and the ability of radiation therapy to enhance tumor immunogenicity provide powerful opportunities for combination multi-checkpoint blockade and adjuvant radiotherapy[116]. Inhibitory checkpoint co-receptors have been shown to be co-expressed on effector T-cells, and at least PD-1 and CTLA-4 are known to signal through distinct and synergistic pathways[20,23,117,118]. Similar to a vaccine, radiation therapy may prime an inflammatory response that induces tumor expression of inhibitory co-receptor cognate ligands which can subsequently be blocked by checkpoint blockade; in addition, radiation causes local tumor destruction, increased immune activity, and an abscopal effect[3,115]. For these reasons, inhibitory immune checkpoint blockade and local radiotherapy are promising components of anti-cancer combination therapy. As discussed, radiation therapy induces the expression of a number of "danger signals", or cytokines and ligands expressed by tumor cells under stress. This stress response is highly immunogenic, and when combined with antibodies that block inhibitory interactions between tumor and T-cell, have the potential to produce significant anti-tumor immune responses. Irradiation of tumor cells up-regulates the retinoid acid early inducible-1 (RAE-1) ligand, which binds to receptor NKG2D on CD8+ T-cells and is critical in the cytotoxic lymphocyte effector response[119]. Another such danger signal released after tumor cell irradiation is CXCL16, which is a chemokine that recruits effector T-cells to the area of inflammation[120]. Sensitizing T-cells within the tumor microenvironment to tumor antigens produces an ideal environment for checkpoint blockade, as evidenced by synergistic responses in metastatic murine breast cancer regression after CTLA-4 blockade combined with radiotherapy[121]. Moreover, preclinical evidence shows that fractionated radiation may be superior to single dose radiation in producing the optimal degree of local inflammation to adequately sensitize T-cells for CTLA-4 blockade[122]. The synergism between radiation therapy and checkpoint

blockade extends to PD-1 inhibition, with significantly increased survival observed in a mouse model of GBM treated with combination anti-PD-1 antibody and radiation therapy compared to either treatment alone[123]. Radiation has been found to up-regulate PD-L1 on tumor cells, and when radiation is combined with antibody-mediated PD-L1 blockade, cytotoxic T-cell activity is increased and MDSC's suppressing effect on T-cells is inhibited[124]. Clearly, preclinical models show that radiation therapy is a potent up-regulator of immune activity in the tumor microenvironment, and has the potential to synergize with checkpoint blockade by sensitizing T-cells to tumor antigens.

Promising outcomes in pre-clinical studies in combination checkpoint blockade of CTLA-4 and PD-1 and combination radiation therapy and CTLA-4 have prompted a slew of clinical trials in an array of solid tumor types. Following from the success of pre-clinical studies combining radiation and anti-CTLA-4 therapy, Slovin and colleagues conducted a phase I/II dose escalation study of Ipilimumab and radiotherapy in castration-resistant prostate cancer (NCT00323882)[125]. At a maximum dose of 10 mg/kg Ipilimumab and 8 Gy of radiation per lesion, 26% of patients experienced grade 3 or 4 adverse events, six out of 33 patients had stable disease and one patient experienced a complete remission. There are now two phase III studies of radiotherapy and Ipilimumab in castration-resistant prostate cancer underway. In addition, a number of phase I and II trials studying combination Ipilimumab and radiation therapy in a variety of solid tumors are currently recruiting or underway, including colon cancer, NSCLC, triple negative breast cancer, melanoma, rectal carcinoma, head and neck cancer, cervical cancer, and metastatic melanoma to the brain. These trials differ in the mode of radiation therapy, varying from stereotactic radiosurgery to whole brain radiation to brachytherapy, and some compare fractionation to single dose. It will be interesting to see whether clinical outcomes correlate to the encouraging findings of pre-clinical studies combining checkpoint blockade and radiation.

Checkpoint blockade and bacterial vaccination vectors

Attenuated strains of *Listeria monocytogenes* has been developed with deletion of the internalin B and actin A genes that are capable of expressing tumor-associated antigens[126]. These strains have been used as tumor vaccination vectors and have been shown to elicit tumor-specific Th1 CD8+ immune responses in murine breast cancer[127-129]. Moreover, Olino *et al*[126] have developed such an attenuated strain expressing antigen AH1 of murine colorectal cancer line CT26; they found that this vaccine successfully treated 90% of mice with hepatic CRC metastases *via* a strong tumor-specific CD8+ response with central and effector memory T-cell generation[126]. Of note, this vaccine down-modulated PD-1 and had variable effects on CTLA-4 on tumor-infiltrating lymphocytes[126]. Given that the anti-tumor effects mediated by *Listeria* vaccines are largely CD8+ mediated, there may be a role for combination *Listeria* and anti-CTLA-4 therapy, as CTLA-4 is expressed on effector T-cells. In a murine model of *Listeria* infection, anti-CTLA-4 therapy after priming with attenuated *Listeria* resulted in an augmented immune responses when mice were re-infected with virulent *Listeria*[130]. Anti-CTLA-4 therapy resulted in increased numbers of CD4+ and CD8+ T-cells specific for *Listeria* antigen, and upon re-infection produced more rapid bacterial clearance. Such data indicates that combination *Listeria* and anti-CTLA-4 therapy could have similar immune effects in a tumor model, priming the immune system with a tumor-antigen expressing *Listeria* vaccine vector and augmenting the resultant Th1 immune response with anti-CTLA-4 antibody. Combination immune checkpoint blockade and bacterial tumor vaccine therapy is a promising future therapeutic that is worth exploring in murine cancer models.

CONCLUSION

In the field of passive immunotherapies, immune checkpoint blockade has garnered significant attention as an efficacious component of anticancer therapy. Anti-CTLA-4 and anti-PD-1 monotherapies have demonstrated significant cancer regression in a number of solid tumor types, but combination blockade of both immune checkpoints has resulted in significant tumor regression[87]. The future of checkpoint blockade may be as an adjuvant to chemotherapy and radiotherapy, augmenting the immune response to destroy even therapy resistant tumor. Research in bacterial vaccine vectors is still in its early stages, but murine cancer models demonstrate robust antigen-specific cytotoxic T-cell responses to *Listeria monocytogenes* vector priming, which can be combined with checkpoint blockade to further augment anti-tumor responses[126,130]. In addition, checkpoint receptor expression in other immune cells, such as NK cells, provides opportunities for inducing anti-tumor immune responses mediated by cells other than T-lymphocytes. KIR is such a checkpoint expressed primarily on NK cells that, when blocked, may prevent downregulation of NK cell activation; moreover, LAG-3 is also expressed in NK cells and other antigen presenting cells[131]. Thus, the future of immune checkpoint blockade may expand to include modulation of many types of immune effector cells in addition to T-cells. The paradigm of anticancer therapy is now making space for immunotherapies, with checkpoint blockade showing great promise for future therapeutics.

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REVIEW

Role of cytokines and other factors involved in the *Mycobacterium tuberculosis* infection

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Abstract

Mycobacterium tuberculosis (*Mtb*) is a pathogen that is widely distributed geographically and continues to be a major threat to world health. Bacterial virulence factors, nutritional state, host genetic condition and immune response play an important role in the evolution of the infection. The genetically diverse *Mtb* strains from different lineages have been shown to induce variable immune system response. The modern and ancient lineages strains induce different cytokines patterns. The immunity to *Mtb* depends on Th1-cell activity [interferon-γ (IFN-γ), interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α)]. IL-1β directly kills *Mtb* in murine and human macrophages. IL-6 is a requirement in host resistance to *Mtb* infection. IFN-γ, TNF-α, IL-12 and IL-17 are participants in Mycobacterium-induced granuloma formation. Other regulating proteins as IL-27 and IL-10 can prevent extensive immunopathology. CXCL 8 enhances the capacity of the neutrophil to kill *Mtb*. CXCL13 and CCL19 have been identified as participants in the formation of granuloma and control the *Mtb* infection. Treg cells are increased in patients with active tuberculosis (TB) but decrease with anti-TB treatment. The increment of these cells causes down-regulation of adaptive immune response facilitating the persistence of the bacterial infection. Predominance of Th2 phenotype cytokines increases the severity of TB. The evolution of the *Mtb* infection will depend of the cytokines network and of the influence of other factors aforementioned.

Key words: *Mycobacterium tuberculosis*, Strains; Virulence; Host genetic; Immune response; T lymphocytes; Cytokines

Core tip: Cytokines are proteins that can alter the behavior or properties of the cell itself or of another cell. These proteins are involved in the immunopathology of different diseases. Study of the cytokines in *Mycobacterium tuberculosis* infection is very important. They participate in the establishment, persistence and

evolution of the infection. The intricate complexity of these regulating proteins stimulate the investigation to the search of more effective treatments that permit the eradication of a disease as tuberculosis which is one of the leading causes of mortality and morbidity worldwide despite efforts made by the scientific community.

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INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) is one of the leading causes of mortality and morbidity in different age groups throughout the world, especially in developing countries. The World Health Organization reported an incidence of 8.6 million cases of TB globally. Most of the estimated number of cases occurred in South-East Asia (29%), African (27%) and Western Pacific (19%) regions. India and China alone accounted for 26% and 12% of total cases, respectively. An estimated 1.1 million (13%) of the 8.6 million people who developed TB were HIV-positive. About 75% of these cases were in the African Region[1]. The latent form of *Mtb,* that represent one-third of the global population, can reactivate years after a primary infection when host immunity declines[2]. In Venezuela the TB prevalence in Warao children was 3190/100.000[3].

Mtb enters the body almost exclusively by the airway (95%). *Mtb* is usually located in the lungs, causing pulmonary TB, but in a variable proportion, can spread through the blood and it produces extra pulmonary tuberculosis, with involvement of the lymph nodes, pleura, genitourinary system, meninges and peritoneum[4].

Pulmonary TB is the main clinical form of the disease and is classified into primary and post-primary (or reactivation). The primary pulmonary TB is due to initial infection with tuberculous bacillus. The location of the primary focus is sub pleural in the mid lung segment. In these primary foci infiltration of lymphocytes, monocytes (MNs) and macrophages (MAs) occur. MAs engulf the bacilli and reach the hilar and mediastinal lymph nodes and occasionally supraclavicular or retroperitoneal causing lymphadenopathy. The injuries of the parenchyma and lymph nodes are resolved spontaneously with calcifications radiographically visible. The post-primary TB is due to endogenous reactivation of the bacillus present in residual foci located in the pulmonary apexes, kidney and/or adrenal glands, which were controlled at the time and remained dormant for many years[4].

Investigations appoint that TB pathogenesis can be divided in four events: inhalation of *Mtb*, inflammatory cell recruitment, control of mycobacteria proliferation and post primary *TB.* Mycobacteria persistence is associated to failure in the immune vigilance; reactivation of the disease, nearby bronchial damage and spreading of the *Mtb* to other areas of the lungs[4-9]. It has been shown that whereas 90% of infected individuals will remain latently infected without clinical symptom, 10% of the individuals infected with *Mtb* will develop active disease[10].

In developing TB, many factors participate, such as: (1) virulence of *Mtb* strain; (2) Mechanisms of *Mtb* Evasion; (3) Host genetic; (4) the coexistence environmental factors such as poverty, malnutrition and overcrowding, facilitate infection; and (5) immune response[11,12].

In this review, we discuss all the factors related with immune response and the participation of cells and regulating proteins in the *Mtb* infection. Also, overall information about the pathological-mechanisms inherent to the behavior of cytokines which allow explaining the clinical manifestations, the evolution of the disease and the resistance to drugs among other aspects, represent a substantial contribution to the knowledge of TB.

IMMUNE RESPONSE, FUNCTION OF THE CELLS AND CYTOKINES PARTICIPANTS IN THE *MYCOBACTERIUM TUBERCULOSIS* INFECTION

Immune response

Many models of animals have been utilized for the study and understanding of TB, such as: Mice[13], rabbits[14], guinea pigs[15], and Nonhuman Primates[16,17]. In addition, studies *in vivo* e *in vitro* in human have provided important insights.

These investigations and other have shown that the balance between host immunity and bacterial evasion strategies among other factors determine the control *in vivo* of *Mtb*. Innate and adaptive immune responses are important for the eradication of the microorganism Figure 1. Pathogen recognition receptors, Toll-like receptors, Nucleotide Oligomerization Domain (NOD)-like receptors, and C-type lectins, have all been implicated in recognition of mycobacteria and in the initiation of the cytokines response. Adaptive immunity is triggered when the bacterial infection eludes the innate defense mechanisms[18]. Gallegos *et al*[19] and Urdahl *et al*[20] have suggested that TB bacterium reside in an immune-privileged site during the earliest stages of infection. Mycobacteria invade the host's pulmonary alveoli, where adaptive immunity is activated. *Mtb* is initially phagocytized by macrophages, where the bacterium is able to survive[21]. Infected macrophages secrete Tumor necrosis factor-α (TNF-α) to recruit CD4+ and CD8+ T cells to the site of infection[22] where they realize effector functions. In turn, cytokines as Interferon-γ (IFN-γ) cause the activation of macrophages[23]. Bacteria are mainly killed by activated macrophages and by cytotoxic

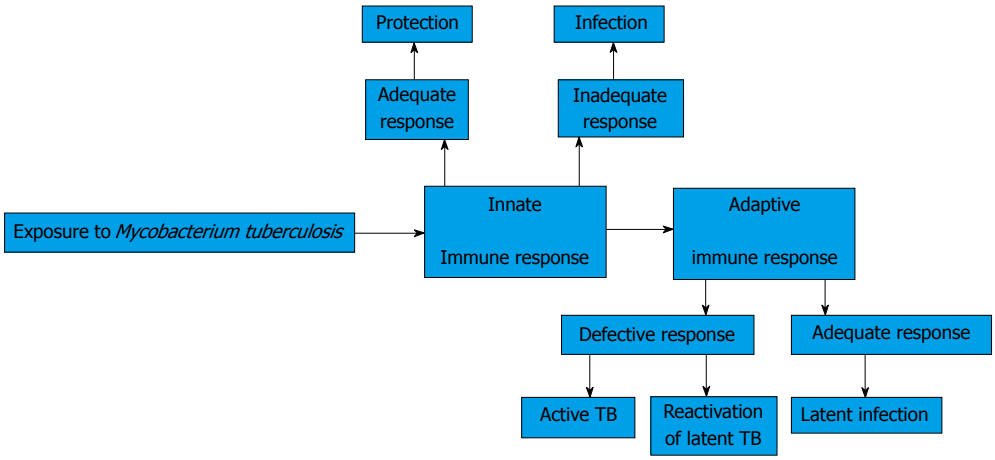

Figure 1 Innate and adaptive immune responses in the *Mycobacterium tuberculosis* infection. TB: Tuberculosis.

functions of activated CD4+ and CD8+ T cells or by TNF-α induced apoptosis of infected macrophages. The balance between pro-inflammatory and anti-inflammatory cytokines regulates the effectiveness of the immune response and tissue damage. Recent studies have demonstrated a role for B lymphocytes towards protection against mycobacterial infections[24]. These lymphocytes form evident aggregates in the lungs of tuberculous humans, non-human primates and mice, which show features of germinal center B cells. These cells can regulate the T cell response, cytokine production and the level of granulomatous reaction[25].

Granulomas form when an intracellular pathogen or its constituents cannot be totally eliminated. These consist of a central core of infected macrophages. The core can include multinucleated giant cells surrounded by epithelial cells. Other cell types are recruited such as: DCs, lymphocytes and fibroblasts. The collagen as element of extracellular matrix integrates the granuloma. This circumscribes the infectious process and also affects immunopathologically sites, where located[26,27].

Matrix metalloproteinases-9 (MMP-9) from epithelial cells initiates recruitment of monocytes to the developing granuloma. During reactivation, granulomas become caseating and necrotic, and the increment of MMP-1 secretion from macrophages allows the degradation of collagen and tissue destruction, which culminates in *Mtb* released into the airways. Experimental studies have revealed the importance of metalloproteinases. Mice treated with an inhibitor of MMPs delayed the formation of granuloma or these were smaller with more collagen. The exact mechanisms by which this balance is achieved, and how it breaks down are unknown. After many years, the organisms emerge from latency to develop post primary tuberculosis that produces cavities in the lungs where the proliferation of large numbers of bacteria occurs and the cough that the patients present facilitate transmission to new hosts[26,27].

Function of the cells

Many cells take place in the immune response in the *Mtb* infection. Also, the regulating proteins that secrete these cells form a complex network that traduces pathological change in cells and tissues. All this determines an active and latent form of the infection which depends of the evolution in time[4].

Phagocytic cells

Neutrophils are among the earliest cells to migrate to the site of *Mtb* infection and evidence exists that these phagocytes participate in the granulomatous reaction[28,29]. Increased neutrophil infiltration has been associated with excessive lung pathology and with poor bacillary control in genetically susceptible mice[30,31]. It has been proposed that neutrophilia is indicative of failed Th1 immunity in response to the use and challenge with aerosol *Mtb*[32]. There is also evidence suggesting that interaction of *Mtb* with neutrophils increment DCs migration to the draining lymph nodes thereby promoting the initiation of adaptive immune response in an aerogenic tuberculous infection[33].

Researchers have evaluated the significance of neutrophils in the protection against *Mtb* and conflicting results have yielded[28,30,34-39]. The role of these professional phagocytes in TB is yet to be clearly defined. However, the roles of neutrophils in development of immune response to *Mtb* could depend on the characteristics of the site of immunological reaction and the level of neutrophilia, as well as other immune system factors[40].

Mtb-induced neutrophil extracellular traps (NETs) were found to be reactive oxygen species and phagocytosis dependent. NETs binding heat shock protein 72 (Hsp72) or recombinant Hsp72 were able to trigger cytokine release from macrophages. NETs can participate in the innate response and influence the immune regulation[41].

The macrophages are activated for many stimuli in the course of an immune reaction and are important in the innate and adaptive immune response. Special attention has been given to macrophages and dendritic cells during the *Mtb* infection. In the pathogenesis of TB,

macrophages are the first-line of defense as the TB bacilli enter the airways[42-45]. *Mtb* has several mechanisms for persisting in human tissues[46-48]. The necrosis of *Mtb*-infected macrophages is considered as the dominant form of cell death instead of apoptosis[47,49,50].

Mtb also promotes its replication by inhibiting the apoptosis of infected macrophages[51]. Apoptosis-associated biomarkers, rather than inflammatory cytokines, are independent factors in predicting active TB. Among the apoptosis-associated biomarkers, Decoy receptor 3 (DcR3) seems to be the most associated with immune cells[52-54]. It has the potential to discriminate between latent and active TB. If 99% of active TB cases can be identified by DcR3 plus PGE2, these both will be useful as a screening criterion[55].

Researchers have demonstrated that *Mtb* suppresses the pyroptosis by macrophages, and possibly in dendritic cells. Pyroptosis is a cell death that is accompanied by the release of pro- inflammatory cytokines from the dying cells and attracts an innate response to the site of infection. This mechanism is different to apoptosis and necrosis[56].

A study has demonstrated that culture conditions can promote or limit replication of the bacteria in macrophages. Also, the cytokines had different effects depending on: the cell period (differentiation or activation), time (early or late) of exposure, concentration of the cytokines, and the magnitude of the microbial challenge. The authors had demonstrated that 40% human plasma, under 5%-10% oxygen, and the involvement of granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α, followed by IFN-γ, limit the replication of the bacteria in macrophages. However, if fetal bovine serum is used, 20% oxygen, GM-CSF, higher concentrations of regulating proteins, and there is premature exposure of IFN-γ, the control of the infection by phagocytic cells is lost. Even, GM-CSF and/or TNF-α contributed with the most successful cellular differentiation, whereas IFN-γ and TNF-α allowed for the best activation. The new culture method will favor the study of antimicrobial mechanisms of human macrophages[57].

Mature dendritic cells (mDCs) are antigen presenting cells. DCs capture Ags of *Mtb* and transport it to the lymph nodes for T cell priming and T helper type 1 polarization because they are important secretors of Interleukin-12 (IL-12) after bacterial stimulation. In contrast, macrophages realize their microbicidal function in the granuloma because they are more efficient in killing intracellular *Mtb*[58,59] and for the maintenance of the Th1 polarity. The IL-12-secreting DCs are considered as the bridge between innate and adaptive immunity in TB, with important implications for DCs-based vaccine designed strategies[60,61]. However; the increment of Cortisol affects significantly the functions of *Mtb*-induced DCs. It has demonstrated a cross-regulation between adrenal steroids and the function of antigen-presenting cells in TB[62].

B lymphocytes

B cells contribute to adaptive immunity by secreting antibodies. Studies have shown that the administration of an *Mtb* high dose in aerosol[63] or intravenous[64] provoke in B cell-deficient mice higher bacterial loads compared to control mice. However, low dose does not alter lung bacterial burden[65-68].

The lungs of *Mtb*-infected B cell-deficient mice display exacerbated inflammation, with enhanced neutrophil recruitment[63]. Experimental evidence suggests that humoral immunity plays a role in the regulation of the Th1 response in TB[69]. It has recently been reported that a subset of B cells (CD19+, CD1d+, CD5+) in the blood of humans with tuberculous infection can suppress pro-inflammatory Th17 phenotype[70].

The lung neutrophilia and enhanced Th17 response seen in *Mtb*-infected B cell-deficient mice could be reversed by passive immune serum therapy, increasing the possibility that immunoglobulins may contribute to the regulation of some immune system cells. Researchers have demonstrated that B cells are required for the development of optimal protective anti-TB immunity upon BCG vaccination by regulating the IL-17/neutrophilic response[40]. The presence of antibody to Ag85 in the sera of TB patients has been associated with a good prognosis[71]. However, studies of B cell immunodeficiency in both humans[72,73] and mice[66,67] have questioned whether these lymphocytes impart a protective effect against *Mtb*.

Investigators based in their results appoint that the participation of B lymphocytes in tuberculous infection is phase-specific. These cells participant in the granuloma formation during the acute infection maintain the local response against *Mtb* and prevent reactivation of the disease during its evolution[29,63,74].

A more recent study has demonstrated that when antibodies interact with stimulatory FCγ receptors of the antigen presenting cells enhance the Th1 response (Predominance of IFN-γ) which controls the infection. Interaction of the antibodies with inhibitory FCγ receptors compromises the anti-bacterial immunity (Predominance of IL-10)[24].There exist immunopathological differences in each case[69]. Other researchers have revealed that the immunity to *Mtb* can be modulated by B cells "in an organ specific manner" with the participation of cytokine production and macrophage activation[75] (Figure 2).

T lymphocytes

Armed effector T cells are crucial to almost all adaptive immune response. Alterations of the Th cells functions conduce to inefficient clearance of pathogens and can cause inflammation and autoimmunity[76].

The reasons for the impaired *Mtb*-specific T cell function in active tuberculosis remain controversial. Patients with mutations in Th-1 cytokine signaling pathways such as IFN-γ and IL-12 (a p40 and p35 heterodimer) are susceptible to overwhelming infection with *Mtb*[77,78]. Impaired Th1 lymphocyte response in *HIV* infection also produces ineffective immunity to *Mtb*[79]. Several observations suggest that the Th2 cytokines, IL-4 and IL-10, are associated with LTB infection, reactivation

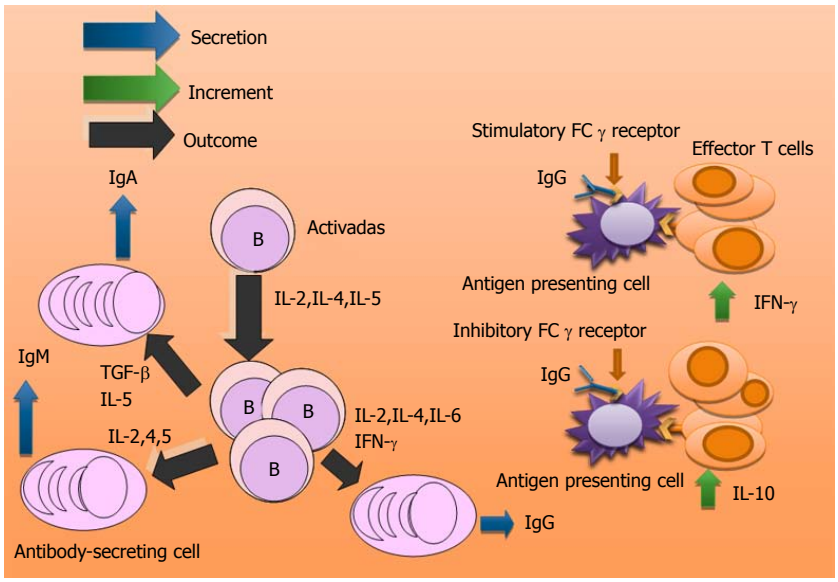

Figure 2 Cytokines involved in antibody secretion in the *Mycobacterium tuberculosis* infection. Interaction of IgG with stimulatory or inhibitor receptors and the secretion of cytokines. TGF-β: Transforming growth factor-beta; IL: Interleukin; IFN-γ:Interferon-γ.

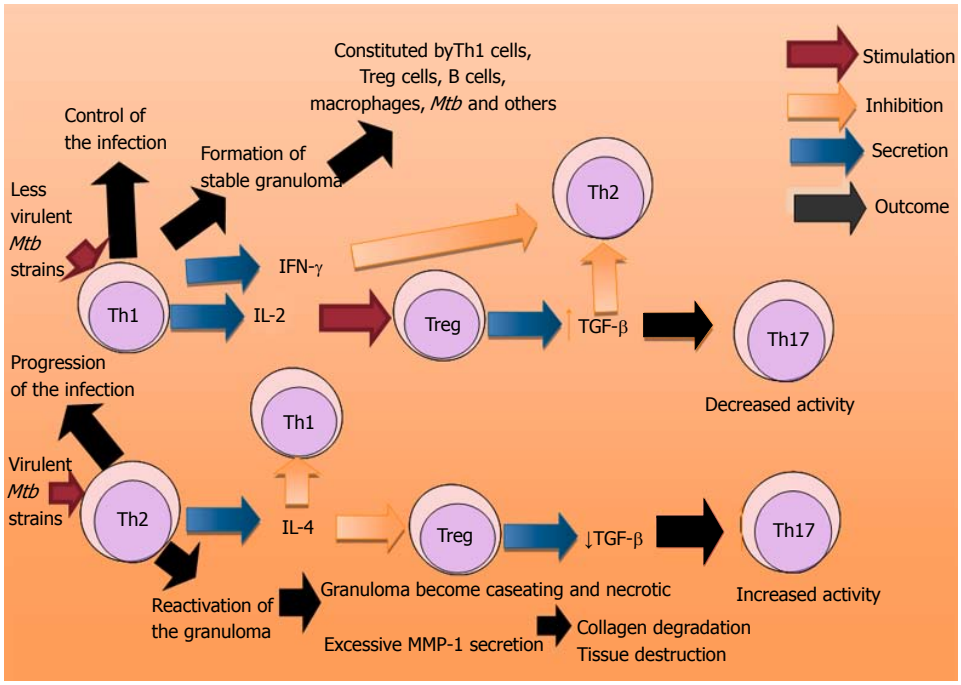

Figure 3 Participation of Th1, Th2, Th17 and T reg phenotypes in the evolution of the *Mycobacterium tuberculosis* infection. TGF-β: Transforming growth factor-beta; IL: Interleukin; IFN-γ: Interferon-γ.

and advanced TB[80,81].

Patients with extrapulmonary disease have immune responses *in vitro* suggestive of Th1 response, whereas patients with miliary/disseminated disease have a suggestive Th2 response[82]. There are several lines of evidence suggesting that overexpression of Th2 cytokines increases the severity of TB, including observations that virulent *Mtb* strains preferentially induce Th2 cytokines expression, whereas less virulent strains induce Th1 cytokines, including IFN-γ and TNF-α[83-85] (Figure 3). There are many factors that can change the immune response in different pathologies, such as: the etiological agent and its immunogenicity, evasion mechanisms of the pathogen, the type of pathology, the phase of the clinical entity, concurrent infections and infestations, the host genetic condition and the sufficiency or insufficiency of the immune system among others[86] (Figure 4). Thereon, authors express that coincident hookworm infection exerts a profound inhibitory effect on protective Th1 and Th17 response in latent TB and may predispose toward the development of active TB in humans[87].

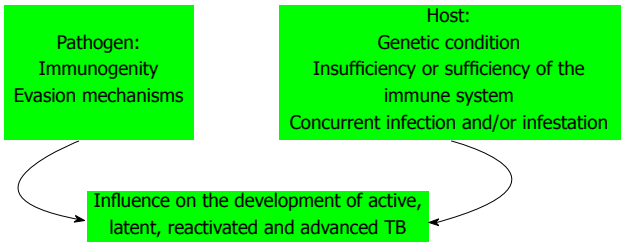

Figure 4 Factors involved in the evolution of *Mycobacterium tuberculosis* infection. TB: Tuberculosis.

In the antigenic presentation the function of MHC class II molecules is to present peptides generated in the intracellular vesicles of B cells, macrophages, and other antigen-presenting cells to CD4 T cells. CD4+ T cells are required for the control of intracellular *Mtb*. The depletion of CD4 T cells increases in quantitative form the bacterial burden associated with MHC II (+/+) cells but not MHC II (-/-) cells[88].

There is no doubt that immunity to *Mtb* depends on Th1-cell activity (IFN-γ and IL-12 and the production of tumoral necrosis factor-α), but Th1 immunity alone is not sufficient to protect the host from *Mtb* infection, development of the disease, or dissemination[8]. For other authors, active TB is characterized by a profound and prolonged suppression of *Mtb*-specific T cell responses, as evidenced by decreased production of the Th1 phenotype cytokines as IL-2 and IFN-γ[89-93]. Overproduction of immunosuppressive cytokines (IL-10 and Transforming growth factor-β) by mononuclear phagocytes has been implicated in decreased T cell function during TB[94-97]. Other studies are controversial with respect to IFN-γ serum levels. These reported in active TB levels significantly higher than in patients during anti- TB therapy, in patients after treatment, in contact and in healthy control. Also, they observed the increment of IL-10, IL-6 and decrease of IL-4[98].

The predominance of Th1 phenotype plays a relevant role in immunity to TB in children. The children are more prone to developing extrapulmonary manifestations of TB than adults. Pediatric TB is characterized by diminished Th1, Th2 and Th17 phenotype cytokines, which favor the development of neurologic TB, suggesting a crucial role for these cytokines in protection against pediatric tuberculosis. Among children with extrapulmonary TB, those with neurologic involvement exhibited a more significantly diminished Ag-driven IFN-γ and IL-17 production[99].

Investigations have implicated Regulatory T cells (Treg) in the pathogenesis of *Mtb* infection. The induced Treg cells (iTreg) are differentiated from naïve T cells in the presence of Transforming growth factor β following T cell receptor (TCR) stimulation. These cells produce large amounts of IL-10 and Transforming growth factor-β[100,101]. Unlike Th1, Th2 or Th17 cells, iTreg displays immune-suppressive activity with minimal antigen specificity[102]. Tregs are increased in the peripheral blood of active TB patients compared with *M. bovis* BCG vaccinated healthy donors. This agrees with recent reports in humans[103] and in the murine TB model[104,105]. It has been demonstrated that Treg cells proliferate and accumulate at sites of infection, and have the capacity to suppress immune responses[105]. Circulating Treg cells in the peripheral blood declined progressively by anti-TB treatment[106].

During the initial T cell response to *Mtb* infection, the pathogen induces the expansions of Treg cells that delay the onset of adaptive immunity, suggesting that *Mtb* has sequestered Treg to allow that the bacterium replicate endlessly in the lungs until T cells finally arrive[107]. The increase of these cells causes down-regulation of adaptive immune response facilitating the persistence of bacterial infections. The induction of Treg by *Mtb* can be an evasive mechanism of the bacterium that permits its replication[108]. Studies have appreciated in active TB infection high levels of circulating Treg cells which inhibit the Th1 response but not the Th17, facilitating the bacterial replication and tissue damage. The presence of persisting immune activation and high frequencies of Treg lymphocytes may reflect immune dysregulation that predisposes individuals to clinical tuberculosis, specifically to extrapulmonary TB[109,110].

CD8 +T cells secrete preformed perforins and granzimes that act over the target cells to die via apoptosis. A study has demonstrated reduced numbers of CTLs expressing low levels of perforin and granulysin, correlated with an elevated frequency of FoxP3+ Treg cells inside of the granulomas. Also, there are high levels of transforming growth factor-β that produce active immunosuppression at the local infection site. These results suggest that an imbalance in the proportion of effector T cells to Treg cells, present at the site of infection, may contribute to the establishment of TB infection[111]. A recent study has identified a mycobacterial protein and peptide recognized by γδ T cells isolated from pulmonary tuberculosis patients. The activated γδ T cells exhibited cytolytic effector function against BCG-infected cells and played a role in the recruitment and activation of other immune cells involved in the antibacterial response[112].

Studies reveal that cytokines network is formed with the participation of the regulating proteins and different subset of cells to achieve control, persistence and severity of TB (Figure 3).

Natural killer cells

Natural killer cells (NK) are important components of innate immune system and mediate resistance against intracellular pathogens. Their cytotoxicity is modulated by a wide variety of cell surface receptors. Both inhibitory and activating receptors encoded by killer immunoglobulin-like receptors (*KIR*) genes bind to HLA ligands to control the activation NK. Not much is known about KIR genes and their influence on the pathogenesis with *Mtb* infection. Activating genes KIR2DS1, KIR2DS5 and inhibitory genes KIR3DL1,

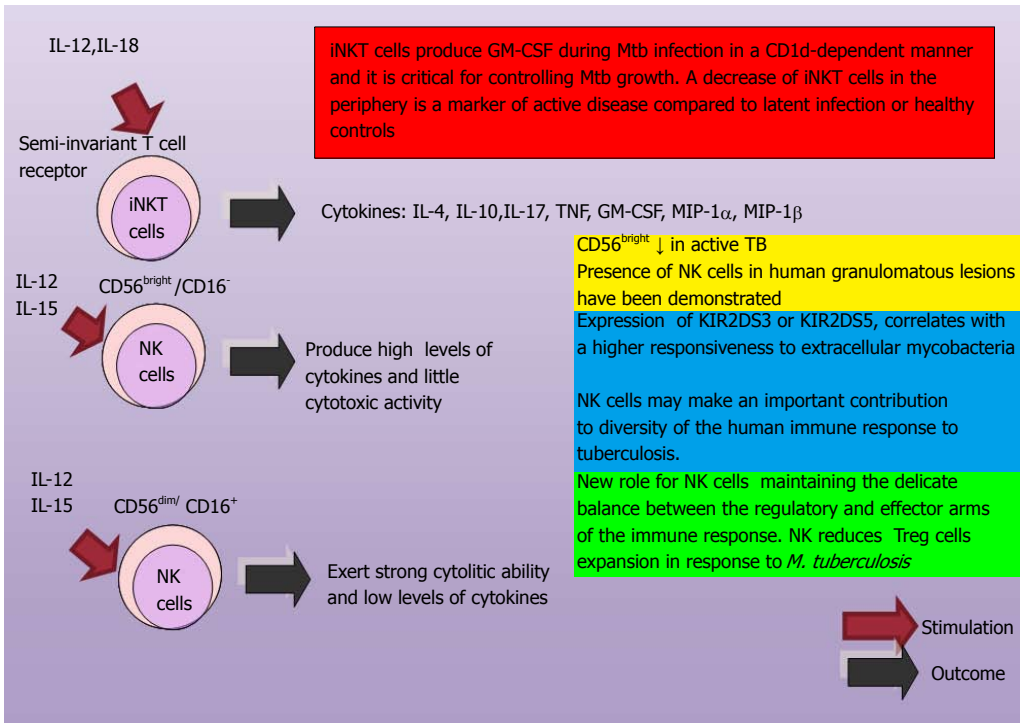

Figure 5 Participation of invariant natural killer T and natural killer cells in the control of *Mycobacterium tuberculosis* infection. iNKT: Invariant natural killer T; CD: Clusters of differentiation; MIP: Macrophage inflammatory protein; GM-CSF: Granulocyte macrophage colony-stimulating factor; TB: Tuberculosis; NK: Natural killer; IL: Interleukin.

KIR2DL3 conferred susceptibility towards TB either individually or in haplotype combinations[113]. A study demonstrated that the aerosol infection with *Mtb*, permit the expansion of the NK cell within the lungs, the expression of markers of activation, and the production of IFN-γ and perforin. These authors appoint that the depletion of NK cells did not affected the bacterial load. Redundant biological actions may be involved[114].

Before, was thought that the memory-like responses were limited to adaptive immunity. Recently, has been demonstrated that NK cells have the capacity for memory-like responses. Three steps have been proposed in the participation of NK cells for the control of infectious processes: initial infection, resolution of inflammation and new inflammatory challenge. An *in vivo* adoptive transfer system was used to determine the NK cell immune memory property. Cytokines secreted by macrophages and dendritic cells induced the production of IFN-γ by NK cells. IFN-γ active CPAs and the naive NK cells transform into memory like NK cells which will be prepared for a new infection and an effective control of the intracellular pathogens such as *Mtb*[115-117]. Investigations have demonstrated that human CD45RO$^+$ NK cells from pleural fluid cells (PFCs) of tuberculous patients express a "memory-like" phenotype that may have an important role in the defense against infection by *Mtb*[118] (Figure 5).

Osteoclasts

Virulent *Mtb* strains that infect multinuclear osteoclasts present an intracellular rapid growth and an osteolytic response, rather than inflammation. Also, highly-fused multinucleated osteoclasts incapacitated the production of cytokines and chemokines[119]. A study reveals that *Mtb* produces a protein called chaperonin Cpn60.1 which stimulates the human and murine monocytes cytokines sintesis. Also, it is a potent inhibitor of osteoclastogenesis both *in vitro* and *in vivo* and is considered a potential cure for osteoporosis[120].

CYTOKINES AND THEIR PARTICIPATION IN THE ANTIBACTERIAL IMMUNE RESPONSE

Cytokines are proteins that participate in regulating the immune system in physiological entities such as pregnancy[121] and other pathologies: bacterial[86,108], viral[122,123], parasitic[124,125], allergic[126,127], rheumatologic[128,129] and neoplastic[130,131], and in deficiencies of Vitamin A and iron[132-134]. Their synergistic, antagonistic, redundant and pleiotropic biological effects can affect or not the immune response against *Mtb.* The cytokines can be regulated for the control of the immune system and the maintenance of homeostasis[86].

Study has provided important details on the *Mtb* lineage-specific patterns of growth and cytokine induction. The lineage 2 *Mtb* strains induce low levels of TNF-α and IL12p40, lineage 3 strains induce high levels of TNF-α, but low levels of IL12p40 and the lineage 4 strains induce high levels of both cytokines[135]. The Modern lineages (lineages 2, 3 and 4) induce lower levels

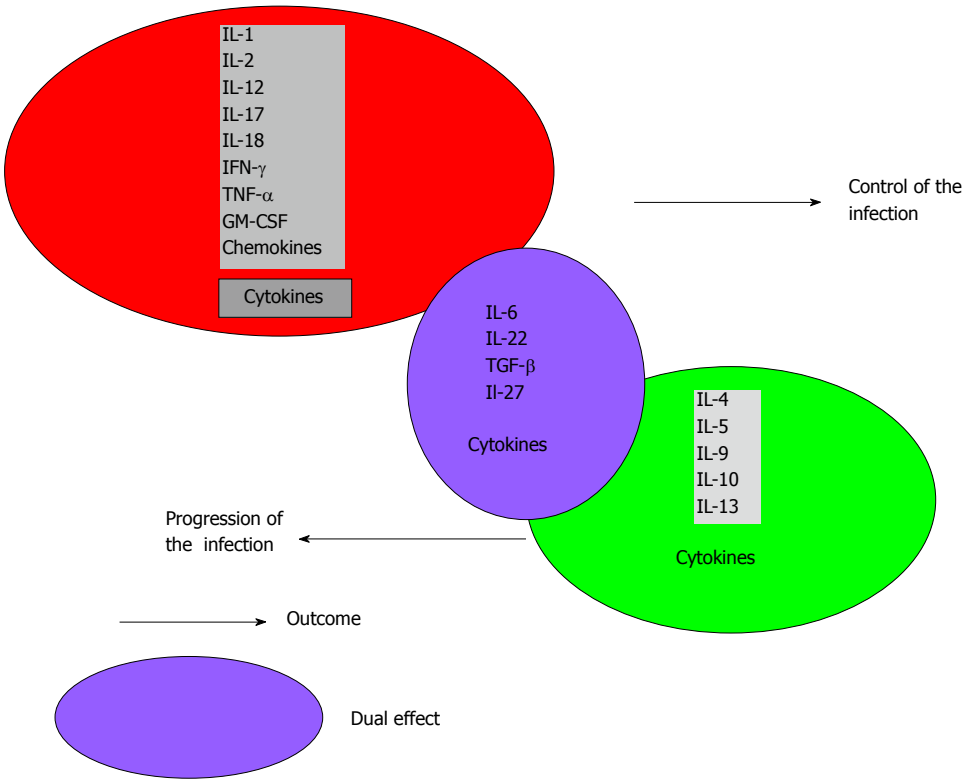

Figure 6 Cytokines in the control and progression of the *Mycobacterium tuberculosis* infection. The concentrations of cytokines, microenvironment and other factors contribute to modify the outcome. TGF-β: Transforming growth factor-beta; GM-CSF: Granulocyte macrophage colony-stimulating factor; IL: Interleukin; IFN-γ: Interferon-γ; TNF-α; Tumor necrosis factor α.

of proinflammatory cytokines when compared with ancient lineages (lineages 1, 5 and 6)[136]. The variability of the immune post challenge response with the strains of those lineages, establishes that studies realized without knowledge of the participant strain, conduces to controversial investigative results.

Strains of *Mtb* influence in the immune response and the evolution of the disease depending of their virulence. Strains of the modern or ancient Beijing (Bj) genotype, as well as the Euro-American lineage, have been used for the induction of ex-vivo cytokine production by PBMCs in healthy individuals. Regarding this, researchers have demonstrated that modern and ancient *Mtb* Beijing genotypes induced different cytokine patterns[137].

Every cytokine, based in its biological actions and interactions with elements of the immune system and other factors, will have a relevant effect in the control or eradication of the *Mtb* infection (Figure 6).

IL-1β

IL-1β directly kills *Mtb* in murine and human macro phages and promotes the recruitment of anti-microbial effector molecules. Also, it augments the TNF-α and Tumoral necrosis factor receptor-1 (TNFR1) cell surface expression and results in activation of caspase-3[138,139]. Vitamin D1, 25-dihydroxyvitamin D (1,25D) directly stimulates *IL1B* gene transcription which is important for macrophage response to *Mtb* infection[140].

Pro-IL-1β maturation is dependent on the NOD-like receptor 3 (NLRP3) inflammasome. IL-1β, in combination with 1,25D, leads to the control of mycobacterial proliferation in the macrophage. 1,25D deficiency is seen in patients with active tuberculosis. This vitamin generally boosts infection-stimulated cytokine/chemokine responses and increases its role in innate immune regulation in humans[141]. Researchers have appreciated a correlation between vitamin D deficiency and TB susceptibility[142,143].

IL-1β is important for host resistance to *Mtb* infection. It has been demonstrated by the significantly reduced survival of IL-1β$^{-/-}$ or IL1R$^{-/-}$ mice after infection[144-147]. Investigations realized in infected infants have shown reduced levels of IL-1 and the affectation of its productive capacity demonstrated immune vulnerability to TB in this population[148-150]. A role for IL-1 in human immunity against TB is supported by several studies showing an association between polymorphisms in the IL-1 or IL-1 receptor genes and host resistance[151-154]. The polymorphisms of *IL-1β* and *IL-10* genes may be valuable markers to predict the risk for the development of TB in household contacts[155]. Studies reveal that mice lacking the signaling adaptor molecule utilized by most membrane-bound TLR (MyD88) are extremely susceptible to TB[156-158]. Significant secretion of IL-1β was detected from macrophages cocultured with NETs from *Mtb*-activated neutrophils[41] (Figure 7).

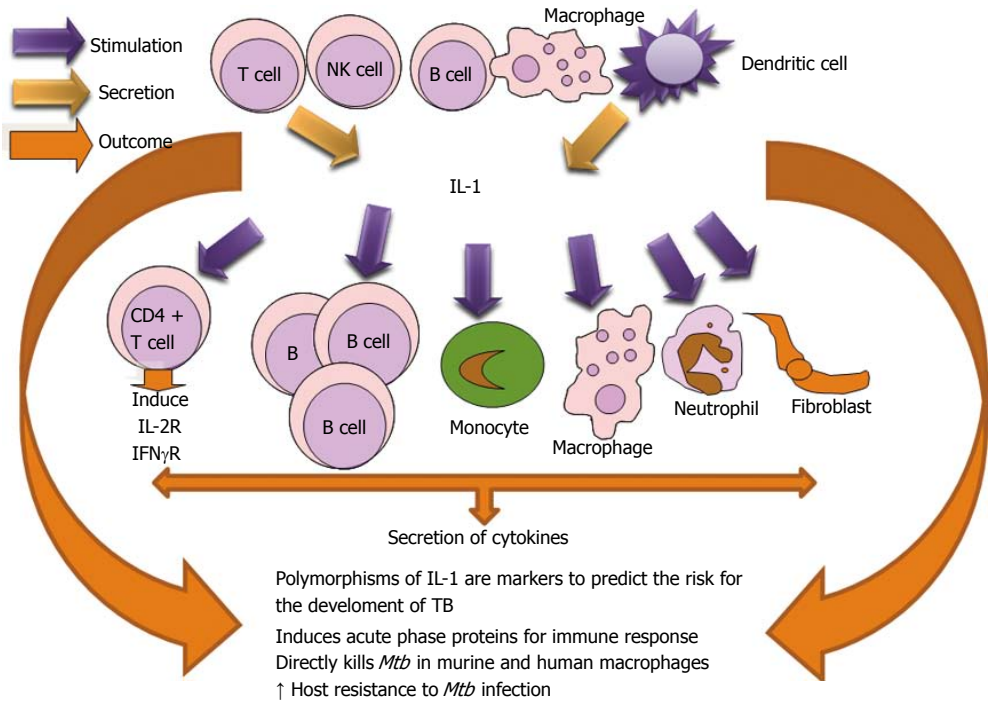

Figure 7 Biological effects of interleukin 1 in the *Mycobacterium tuberculosis* infection. TB: Tuberculosis; NK: Natural killer; IL: Interleukin; CD: Clusters of differentiation; *Mtb*: *Mycobacterium tuberculosis*; IFN: Interferon.

TNFα

TNFα is produced by the Th1, some Th2, and some CTL phenotypes. It induces nitric oxide production and activates microvascular endothelium among other biological actions. It is a cytokine whose deregulated expression may cause immunopathology[159,160]. However, in countries with a high incidence of TB, the biological therapy with anti-TNF-α has been associated with immunosuppression, reactivation of latent TB[161-163] and a risk of new *Mtb* infection[164,165].

The exacerbation of TB occurs with the breakdown of Granuloma which has an important role in the host protection against mycobacterial infections[166-168]. However, the host immunity can decline and provide chance to reactivate the latent form in the granulomas which can be a niche where mycobacteria might persist[169]. *Mtb* induces exacerbated inflammatory responses associated to important tissue lesions and dissemination of the bacilli into the airways[170].

Dysregulated TNF expression has been associated with defective host immunity due to excessive or inefficient inflammation[171]. In HIV patients with pulmonary TB, a clinical trial combining TNF inhibitors with anti-TB drugs showed that TNF inhibitors can be safely administrated during TB treatment and, in addition, higher responses to TB treatment were observed in the group of Enbrel (etanercept, soluble TNFR2-Fc) treated patients[172].

Mtb chemotherapy may be more efficient in the presence of a TNF inhibitor to clear bacilli and reduce lung pathology, which may be considered in acute and chronic *Mtb* infection[173]. However, for other authors the neutralization of TNF α produces disseminated disease in acute and latent *Mtb* infection without alterations in the granuloma structure in a cynomolgus macaque model[174]. TNF expression is necessary for controlling *Mtb* infection *in vivo* and TNF neutralization in monkeys and humans correlates with an increased risk of reactivation of latent tuberculosis[161,174].

Infection of human alveolar macrophages by *Mtb* has been reported to be sufficient to induce apoptosis mediated by TNF-α in an autocrine/paracrine manner and proinflammatory cytokines directly or indirectly modulated apoptotic response depending on the degree of virulence of the strain[175]. It has been observed that serum TNF α and Malondialdehyde (MDA) measure ments may play an important role in the evaluation of the inflammatory phenomena in TB[176]. Also, a positive correlation was found between an increase in serum TNF-α levels and clinical deterioration in patients with a severe form of TB[177] (Figure 8).

IL-2

IL-2 is produced by Th0, Th1 and some CTL. It stimulates growth of B, T and NK cells and is essential for cellular immunity and granuloma formation in *Mtb* infection. The IL-2 liberation is stimulated by TB-specific antigens and was significantly higher in TB patients than in healthy controls and suggested that IL-2 could be a potential biomarker for diagnosing TB[178-180]. The detection of IL-2 and IFN-γ permits to discriminate between active and latent tuberculosis when compared with controls[181]. Another study did not appreciate the utility of the IL-2 as a diagnostic biomarker for TB

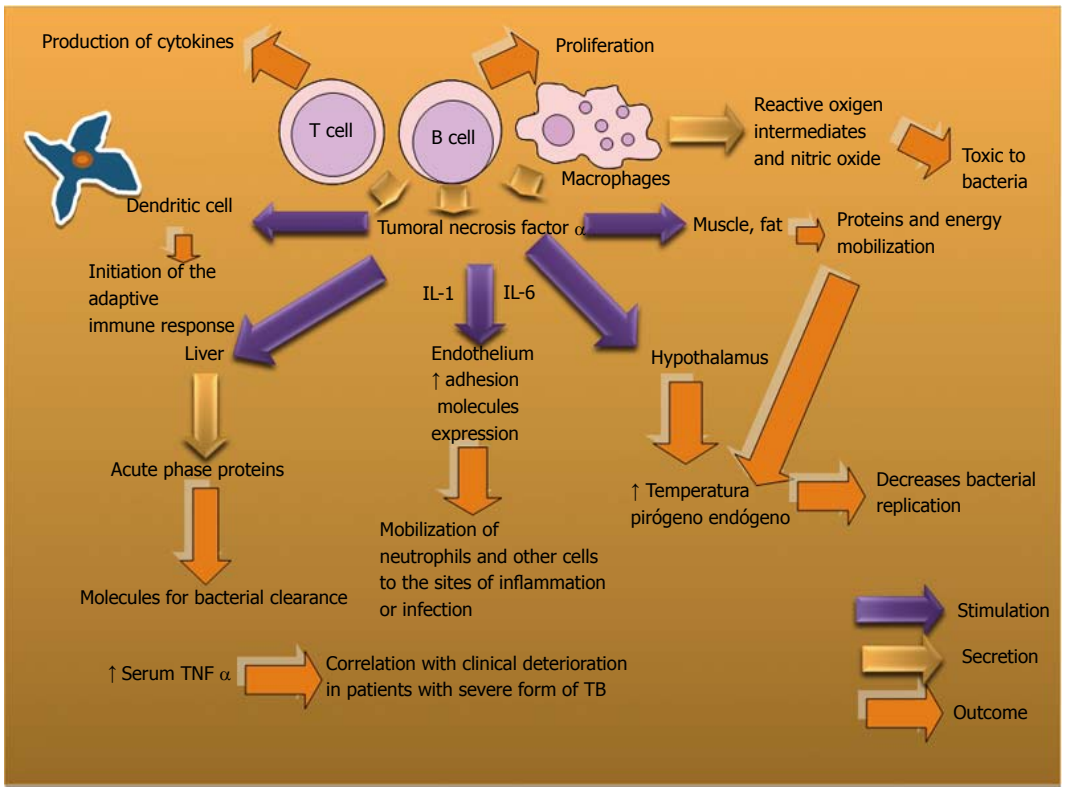

Figure 8 Biological actions of Tumor necrosis factor α to control *Mycobacterium tuberculosis* infection. TB: Tuberculosis; IL: Interleukin.

infection due to its low amount released[182]. Studies have reported that IL-2 and IL-9 expressions are elevated in PBMC (Stimulated by ESAT-6) from TB patients[183,184]. Researches demonstrated that immunotherapy with both IL-2 and GM-CSF may be useful to treat multidrug resistant tuberculosis (MDR-TB). Mice receiving immunotherapy developed fewer lesions in the lungs compared with mice receiving antibacterial therapy alone[185] (Figure 9).

IL-4

IL-4 is produced by lymphocytes Th2 and activated mast cells. This cytokine stimulates the IgG4 and IgE isotype change, acts as an autocrine growth factor for Th2 lymphocytes, also, inhibit the development of Th1 and Th17 lymphocytes and participates in the activation of macrophages[76,186].

Increased production of the Th2 cytokine (IL-4) by bronchoalveolar lavage cells (BAL) is a strong risk factor for TB transmission in South African patients. Increasing IL-4 was associated with BAL PMNs and negatively associated with BAL lymphocytes. IL-4 has been implicated in conversion of LTB infection to active TB[187]. IL-4 has been postulated as key in TB pathogenesis, especially with its ability to down-regulate inducible nitric oxide synthase, Toll-like receptor 2, and macrophage activation[188].

Clinical studies involving patients with latent TB show a clear correlation between the intensity of a Th2 response and the risk of developing active disease and in particular a direct correlation between the level of IL-4 messenger RNA and disease severity[189]. The induction of IL-4 production by DCs generated by BCG-infected monocytes could explain the failure of the BCG vaccine to prevent pulmonary TB[190]. It has been demonstrated that high levels of IL-4 were associated with disease progression in TB-susceptible families when there is lack of IFN-γ expansion. Resistant families have overrepresentation of IFN-γ +874 A allele and an increment of IFN-γ secreting cells[191]. Authors agree in relation to the worsening of the host immune response to *Mtb* due to the effects of IL-4[191,192]. However, the mechanisms of inhibition may be different. Inhibition of autophagy through the autocrine secretion of IL-4 and/or IL-13 by infected macrophages could allow that the bacteria gain a foothold previous to the formation of a protective granuloma[193]. This effect on the autophagy has been demonstrated in murine and human macrophages[194] (Figure 10).

IL-5

IL-5 is produced by Th2 phenotype. It activates to eosinophil and stimulates its growth and differentiation. Also, it stimulates proliferation of lymphocytes and synthesis of IgA antibody[195,196]. This regulating protein may be a factor in the reduction of *Mtb*-specific T cell responses within coinfected (SIV/Mtb) individuals. Researchers found that neutralizing IL-5 in coinfected monocytes partially restored normal T cell TNF production[197]. The presence of eosinophils in the cellular infiltration at the site of mycobacterial infection strongly suggests that increased levels of IL-5 are

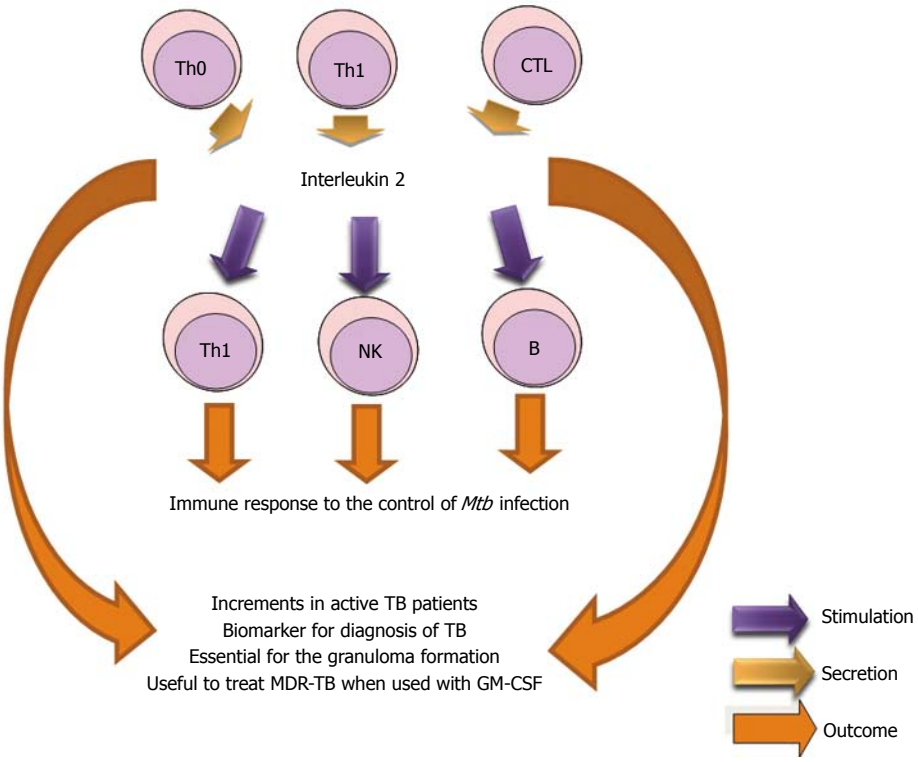

Figure 9 Role of IL-2 in *Mycobacterium tuberculosis* infection. TB: Tuberculosis; MDR-TB: Multidrug resistant tuberculosis; GM-CSF: Granulocyte macrophage colony-stimulating factor; NK: Natural killer; MDR-TB: Multi drug resistant tuberculosis.

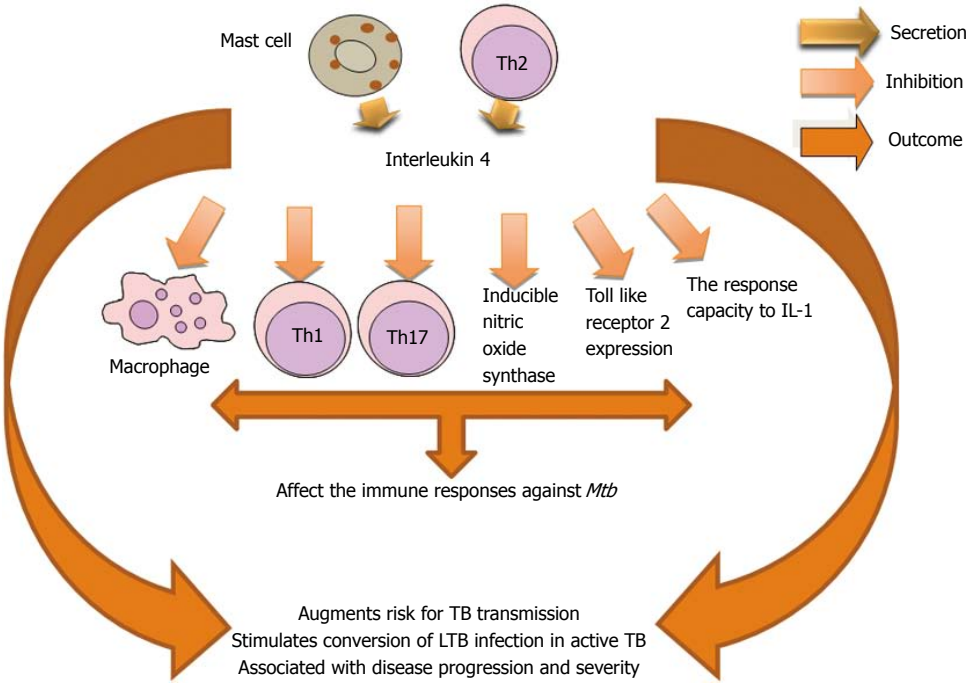

Figure 10 Participation of interleukin-4 in *Mycobacterium tuberculosis* infection. TB: Tuberculosis; LTB: Latent tuberculosis.

produced *in vivo* during *Mycobacterium bovis* bacillus Calmette Guérin (BCG) infection in the absence of IFN-γ signaling[198,199] (Figure 11).

IL-6

IL-6, IL-1 and TNF-α, are important inductors of the acute-phase response. These cytokines are termed endogenous pyrogens because they cause fever and derive from an endogenous source rather than from bacterial components. IL-6, together with the other cytokines aforementioned, has effect on hepatocytes, Bone-marrow, endothelium, hypothalamus, fat, muscles

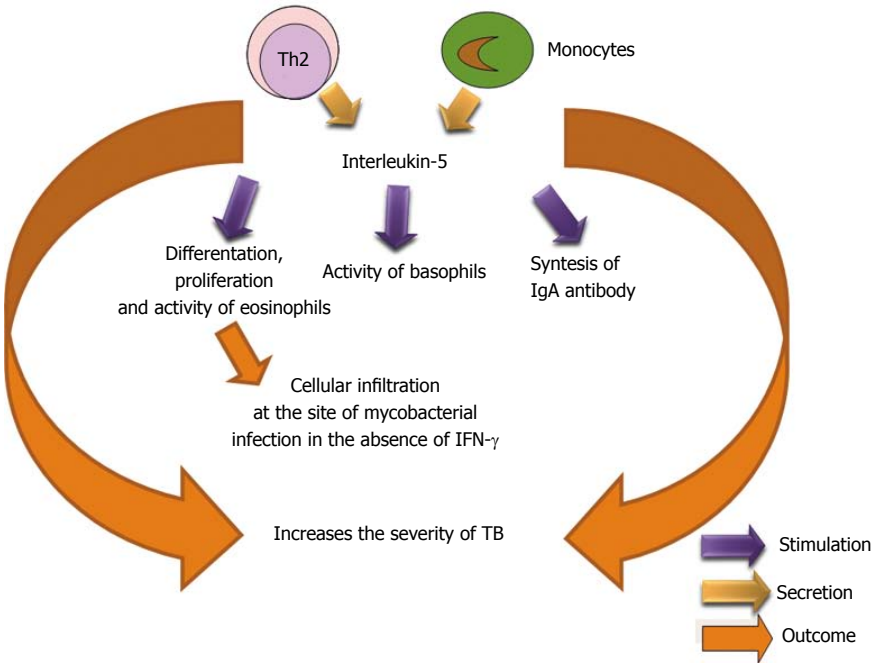

Figure 11 Participation of interleukin-5 in the *Mycobacterium tuberculosis* infection. TB: Tuberculosis; IFN-γ: Interferon-γ.

and DCs[200].

It has been reported that IL-6 plays an important role in protection against murine *Mtb* infection[201,202] due to the influence of the $CD4^+$ T cells response[203]. *Mtb*-infected IL-6-deficient animals show an impaired Th1 response and increased bacterial loads, indicating a requirement for IL-6 in host resistance to *Mtb* infection[201,204]. IL-6 down regulates macrophage microbicidal activity and IL-6 inhibits the production of INF-α and promotes *in vitro* growth of *Mycobacterium avium*[205,206]. IL-6 secreted by *Mtb*-infected macrophages suppresses the responses of uninfected macrophages to IFN-γ[207]. Increased levels of IL-6 in the lungs, along with increased levels of IL-1β and IL-11, is significantly correlated with tuberculosis progression in genetically susceptible mice[208]. Together, these mice studies indicate that IL-6 may play multiple roles and contribute both positively and negatively to host control of *Mtb* infection.

IL-6 has been shown to contribute to the differentiation and activation of cells of the immune response and others not related with this system[209]. It is associated with the pathogenesis of many chronic inflammatory diseases, including tuberculosis[208,210,211]. Genetic variants in *IL-6/IL-6R* have been linked to the susceptibility to the severity of a wide range of diseases, such as: respiratory tract infection, asthma, meningococcal disease, chronic hepatitis C virus infection and rheumatoid arthritis[212-216]. A rare genetic variation in the *IL-6* gene, rs1800796, is significantly associated with tuberculosis disease in the Chinese Han population[217]. Down regulation of IL-6R expression on CD4 T cells in patients with active pulmonary TB is associated with decreased of Th17 phenotype response, suggesting a role for IL-6 in the progression of TB in humans[217-219].

Lung parenchyma can be destroyed during active TB and it provokes immunological alterations for controlling the infection. Patients with radiographically advanced TB showed an increase of the inducible protein-10 (IP-10) and IL-6 production by BAL cells and these are biomarkers of non-cavitary TB. This may reflect an effective Th1 immune response for controlling TB and for attenuating the tuberculous lung destruction. The patients with lung cavities had a higher percentage of polymorphonuclear neutrophils (PMN) in BAL as well as lower IP-10 and IL-6 compared to those without cavities. Also, was demonstrated a negative association between IP-10 and PMN of BAL[220] (Figure 12).

IL-9

It is known that Th9 phenotype cells secrete the regulating proteins IL-9 and IL-10. Th9 cells are involved in the intestinal responses to helminths which were thought to be mediated only by the Th2 phenotype. Studies have demonstrated that the increased expression of IL-9 may contribute to the development of TB and it is associated with an impaired Th1 immune response in patients with tuberculosis[184,221]. Th9 cells with the phenotype of effector memory cells were found in tuberculous pleural effusion as compared with blood. Pleural mesothelial cells were able to function as antigen-presenting cells to stimulate Th9 cell differentiation. Further investigations are needed to reveal the function of this type of cells and their products in pathogen clearance and inflammatory diseases[76,222] (Figure 13).

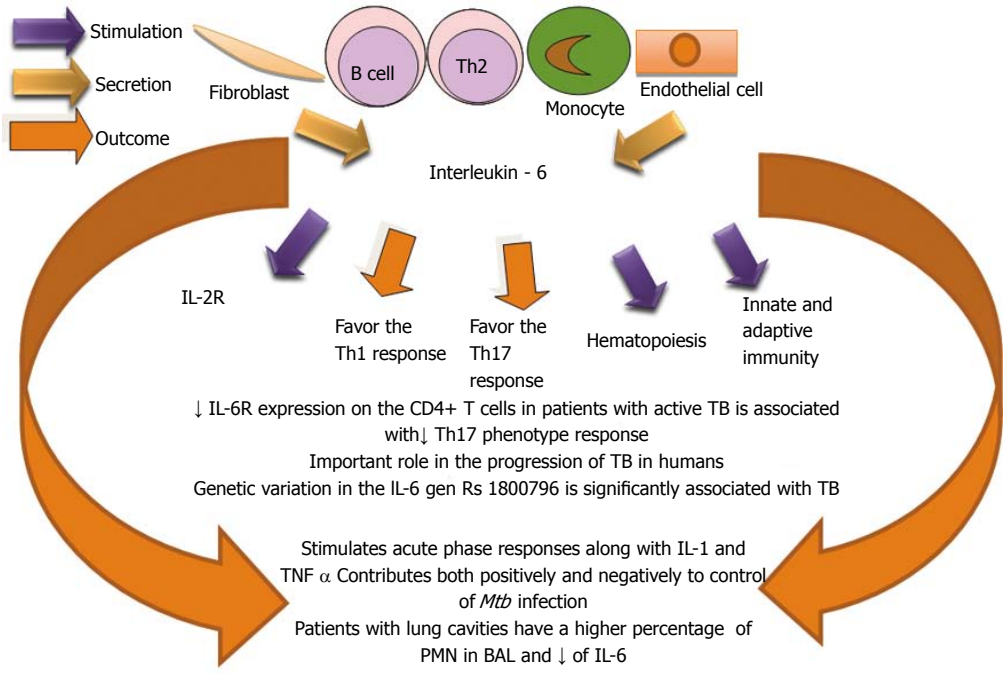

Figure 12 Role of interleukin-6 in the *Mycobacterium tuberculosis* infection. TB: Tuberculosis; PMN: Polymorphonuclear neutrophils; IL: Interleukin.

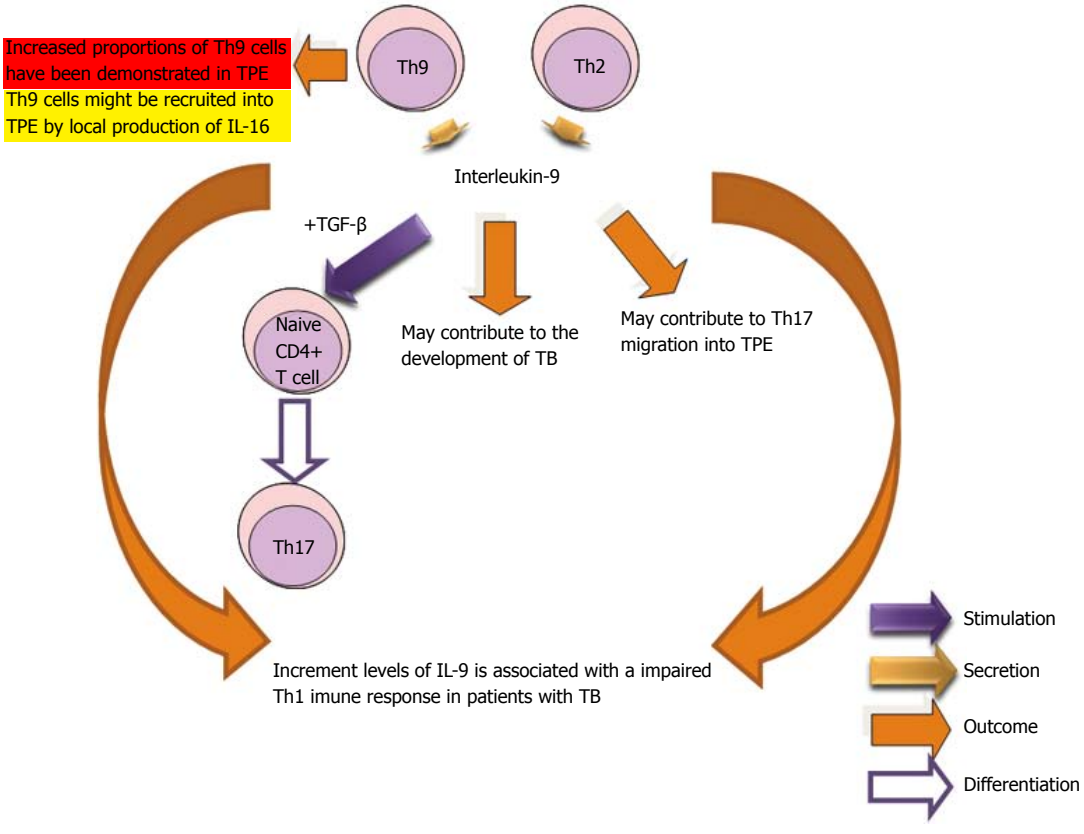

Figure 13 Participation of interleukin-9 in the *Mycobacterium tuberculosis* infection. TB: Tuberculosis; TPE: Tuberculous pleural effusion; TGF-β: Transforming growth factor-beta; CD: Clusters of differentiation; IL: Interleukin.

IL-10

IL-10 is an immunosuppressive cytokine that is produced by Th0, Th1, Th2 and T regs phenotypes among other cellular types. It inhibits Th1, augment MHC class II and gene knock-out of this regulating protein cause inflammatory bowel disease. IL-10 and transforming growth factor-beta (TGF-β) restrict T effector cell response[223]. Increase in $CD4^+CD25^+$ $FoxP3^+$ cells has

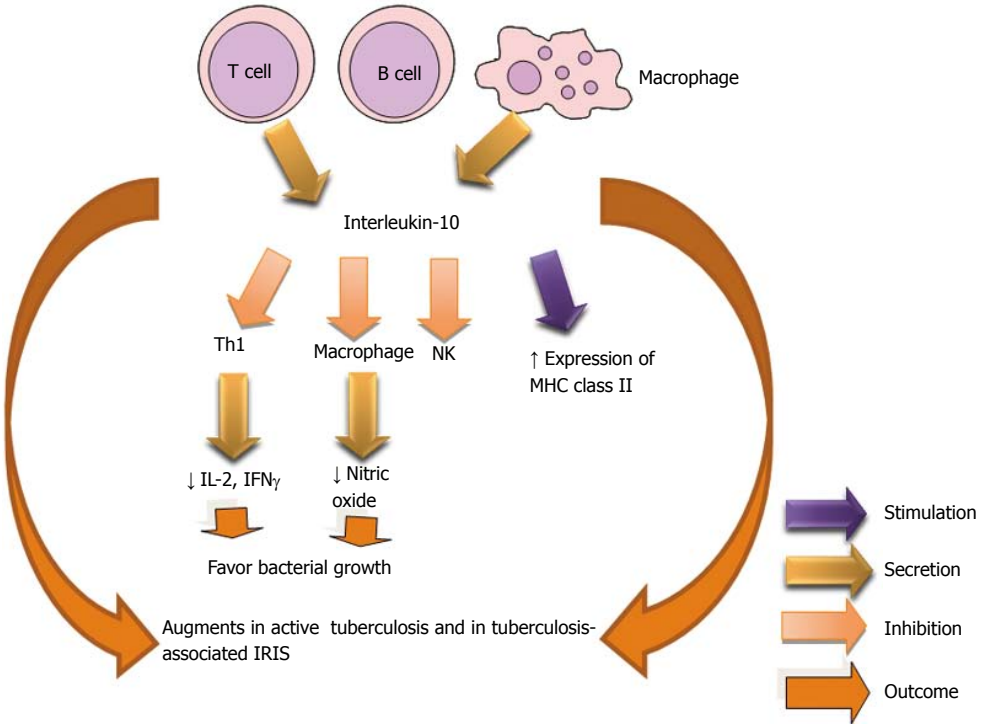

Figure 14 Influence of interleukin-10 in the control of *Mycobacterium tuberculosis* infection. IRIS: Immune reconstitution inflammatory syndrome; IFNγ: Interferon γ; NK: Natural killer; IL: Interleukin.

been shown to decrease Th1 cell responses in patients with TB[224]. IL-10 has been reported to modulate the innate and adaptive immune responses, potentially creating a favorable environment for the persistence of microbes, intracellular pathogens, and chronic infections[225]. The increased ability of macrophages to produce IL-10 when stimulated with Toll-like receptor ligands is also associated with an increased tendency to develop primary progressive tuberculosis[226]. Production of IL-10 has also been reported to be higher in patients who had active TB, compared with tuberculin skin test responders[227].

IL-10 plays an important role in Mtb infection, where the cytokine has shown to reduce the immunity. IL-10$^+$ T cells with immunosuppressive properties are present in anergic TB patients[228]. IL-10 decrease the macrophage activity in the Mycobacterium avium infection and the administration of monoclonal anti-IL-10 diminishes bacterial growth in the spleen[229]. IL-10 helps maintain mycobacterial infections[230]. It has been demonstrated *in vivo* that the production of IL-10 reactivates the chronic pulmonary tuberculosis[231]. The heterogeneity of macrophages may be determinant in disease outcome in intracellular bacterial infection as type I and II macrophages have opposite effects in the cellular immunity[232].

Study revealed an increase in the transcript levels of IL-10 and IL-22 in tuberculosis-associated immune reconstitution inflammatory syndrome (IRIS) patients, compared with non-IRIS controls. The serum samples showed statistically significant high concentrations of IL-10 and IL-22 cytokines in tuberculosis-IRIS patients[233]. Two forms of TB-IRIS are recognized: paradoxical and unmasking. The first manifests with new or recurrent TB symptomatology and second with an exaggerated and unusually inflammation. Both forms have occurred during the early anti retroviral therapy[234] (Figure 14).

IL-12 and IFN-γ

IL-12 is crucial for optimal differentiation and main tenance of IFN-γ-secreting antigen-specific Th1 cells[235,236], and in controlling mycobacterial infections in mice and men[237,238]. The increase of IL-12p40 production by BAL cells in sputum of patients with radiographically advanced TB reveals less effective immune control and more complications. It has been demonstrated that IL-12 receptor deficiency is found in healthy individuals with mycobacterial infections[77,187].

Parenteral administration of IL-12p70 to *Mtb*-infected IL-12p40-deficient mice restores CD4$^+$ T-cell production of IFNγ and control of bacterial growth in the lungs and spleen, whereas these effects are lost when administration of IL-12p70 is discontinued[235].

Researchers appoint that IL-12 enhanced the expression of granzyme B, activation inducer molecule (CD69), IL-2 receptor α chain (CD25), Natural Killer Group 2D (NKG2D), IL-12 receptors β1 and β2 on CD45RO$^+$ NK cells from pleural fluid cells (PFCs) from tuberculous patients. Also, they have demonstrated that CD45RO$^+$ NK cells produced significantly more IFN-γ and were more cytotoxic compared with CD45RO$^-$ NK

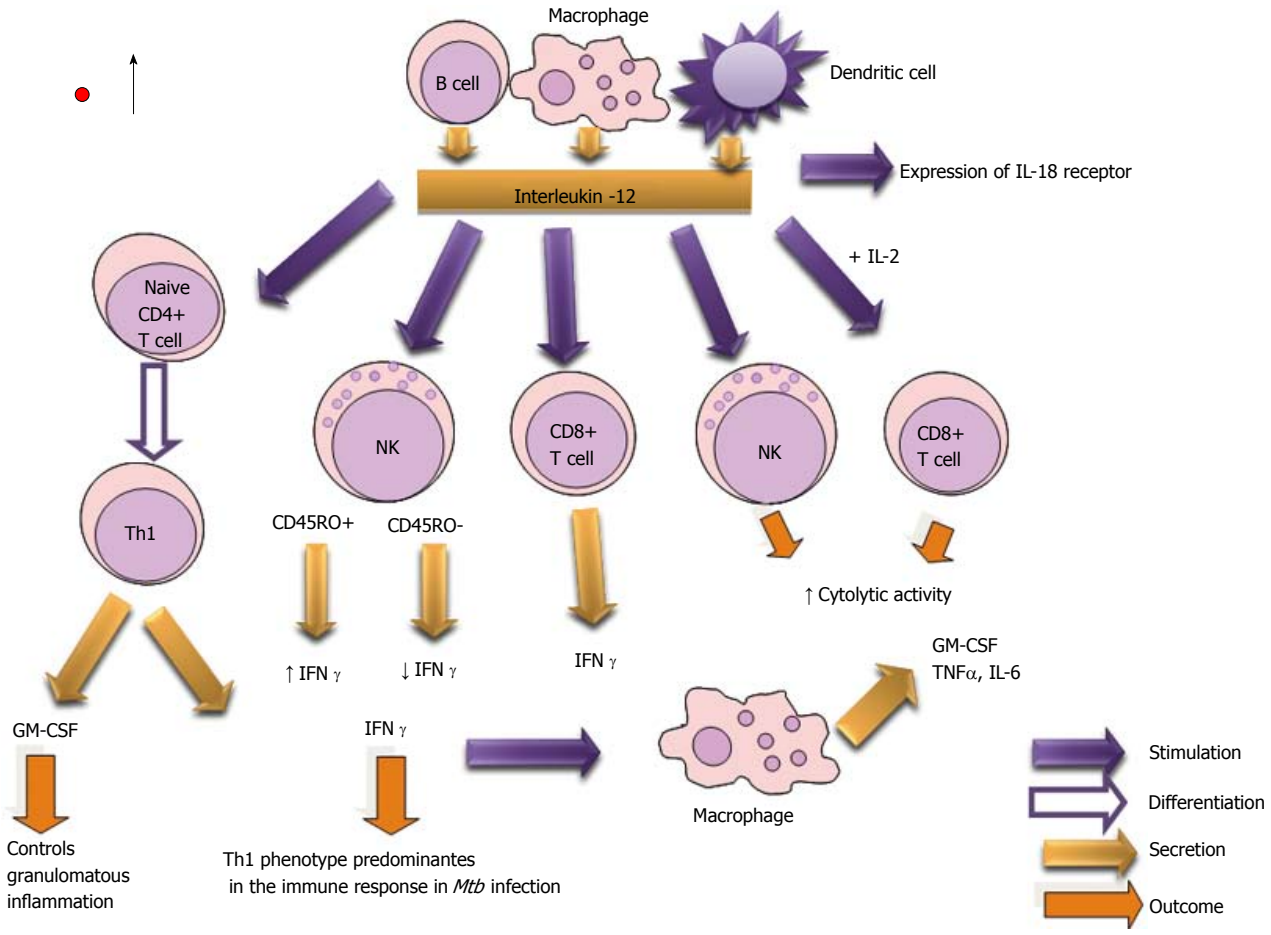

Figure 15 Biological actions of interleukin-12 to control of the *Mycobacterium tuberculosis* infection. GM-CSF: Granulocyte macrophage colony-stimulating factor; NK: Natural killer; CD: Clusters of differentiation; IL: Interleukin; IFN γ:Interferon γ.

cells from PFCs when stimulated with 12 IL-12. The activity of NK cells is associated with early resistance against *M. tuberculosis* infection[118,239] (Figure 15).

Another pro-inflammatory cytokine is IFN-γ which is secreted by Th1, CTL and NK cells. It participates in the synthesis of IgG2a, inhibits the phenotype Th2, activates NK cells and augment MHC class I and II. Also, the gen knock-out produces susceptibility to mycobacteria. IFN-γ has been shown to be an important mediator of macrophage activation involved in the control of a number of intracellular pathogens[240-247].

The active tuberculosis is 5-10 times more frequent in infants than adults. Also, the children have higher rates of severe disseminated disease. It has been shown that infant T cells are less capable of transforming into IFN-γ -producing T cells[244]. IFN-γ stimulated responses are lowered in TB, while the expression of Suppressor of Cytokine Signaling (SOCS) molecules-1 and 3 and $CD4^+CD25^+FoxP3^+$T regulatory cells are increased[247]. The enhanced susceptibility to mycobacterial infection of IFN-γ knockout mice[246,247], and of patients with genetic defects in IL-12/ IFN-γ pathway[248], provides strong evidence that IFN-γ is required in defense against *Mtb*.

BCG is a licensed vaccine in use that mediates immune protection through the production of IFN-γ by CD4 T cells, which activates macrophages to kill *Mtb*. However, some recent studies have reported a lack of correlation between IFN-γ production by CD4 cells and BCG-induced immune protection[249]. IFN-γ is necessary for the control of TB[23,250] and has been the focus of multiple coinfection studies. These studies conclude that HIV reduces IFN-γ production by *Mtb*-specific CD4 T cells in the periphery and airway[251-254].

Study reveals that dehydroepiandrosterone increments the antigen-specific T-cell proliferation and IFN-γ production induced by *Mtb*-stimulated DC. The adrenal axis is important in the modulation of the DCs function in the context of TB[62,255]. Mice deficient in IFN-γ ($IFN\text{-}\gamma^{-/-}$) or the IFN-γ receptor ($IFN\text{-}\gamma R^{-/-}$) are extremely susceptible to infection with tuberculosis-causing organisms[23].

Murine macrophage studies show that IFN-γ induces *Mtb* killing[23,255] but when *IFN*-γ gene has been disrupted is unable to contain or control sublethal dose of *Mtb*[256].

The secretion of IFN-γ and, to a lesser extent, of IL-17 by CD4 (+) T cells plays a major role both in protection and immunopathology[257]. But the effect of IFN-γ in human macrophages remains controversial[258,259]. Regard this, some researchers have shown that IFNγ

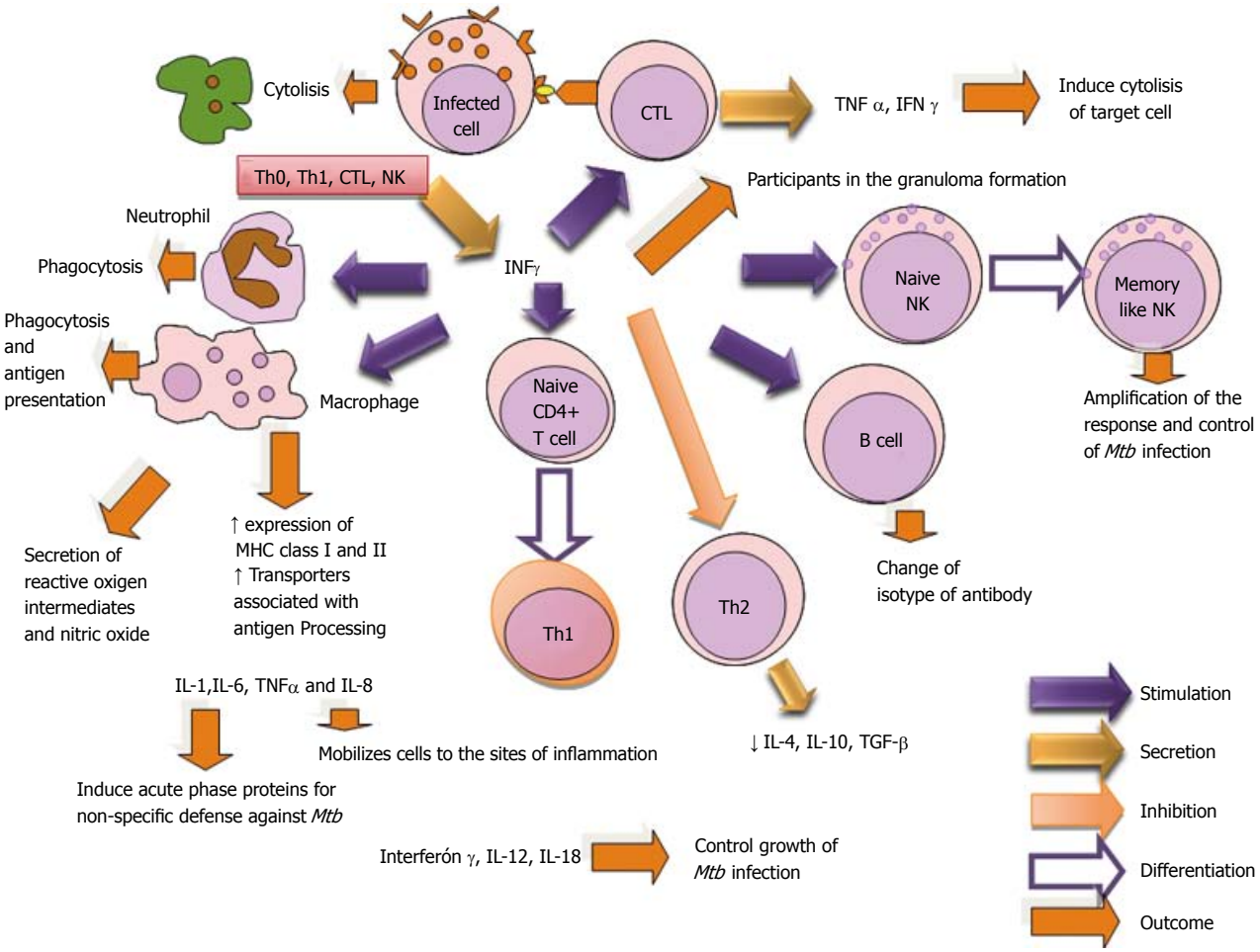

Figure 16 Biological actions of Interferon-γ in *Mycobacterium tuberculosis* infection. TGF-β: Transforming growth factor-beta; NK: Natural killer; CTL: Cytotoxic t lymphocyte; CD: Clusters of differentiation; IL: Interleukin; CTL: Computation tree logic; Th1: T helper type 1.

activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria[260]. For others the IFN-γ-mediated anti mycobacterial activity requires specific *in vitro* conditions for human macrophages, such as physiological O_2 levels and the presence of the GM-CSF[57]. Extracellular trap formation and mycobacterial aggregation are IFN-γ-inducible events and require the ESX-1 secretion system. In the absence of ESX-1, IFN-γ does not restore any extracellular trap formation, mycobacterial aggregation, or macrophage necrosis[261]. Therefore, the monitoring of the *in vivo* and *in vitro* metabolic activity of both slow-growing and fast-growing mycobacteria, using methods as chronoamperometry and chronopotentiometry is of great importance[262].

Th1 phenotype plays a relevant role in the formation of granulomas. IFNγ is the most characteristic cytokine produced by armed Th1 cells (Figure 16).

IL-13

IL-13 is produced by T lymphocytes and exerts its biological functions on B cells and monocytes, and inhibits pro- inflammatory cytokines production. It upregulated MHC class II expression, also, promotes IgE class switching. This cytokine is a key regulator of the extracellular matrix, and is redundant with IL-4. Concentrations of IL-13 were found to be significantly higher in fast responders to antimycobacterial treatment than in slow responders in the fifth week of treatment. The role of IL-13 in *Mtb* infection is not well defined. IL-13 abrogates autophagy-mediated killing of *Mtb* in human and murine macrophages[194]. However, IL-13 has modulated the resistance to a number of intracellular pathogens including *Leishmania major*, *L. mexicana* and *Listeria monocytogenes.* The elevated level of IL-13 observed in fast responders compared with slow responders may suggest their better resistance to the infection, although the mechanisms for the IL-13 effect are not yet clear[263]. IL-13 can be substituted for IL-4 in several physiological responses. However, the presence of IL-13 inhibits the action of IL-4 on *Mtb*-induced IL-8 secretion but does not affect the inhibition of IL-8 secretion by IL-10[264].

IL-4 and IL-13 are well recognized as activating distinct signaling cascades[83,265-268]. IL-13 inhibits IFN-γ-induced autophagy, but this process is independent of protein kinase B (AKT); instead it is dependent of the signal traducer and activator of transcription 6.

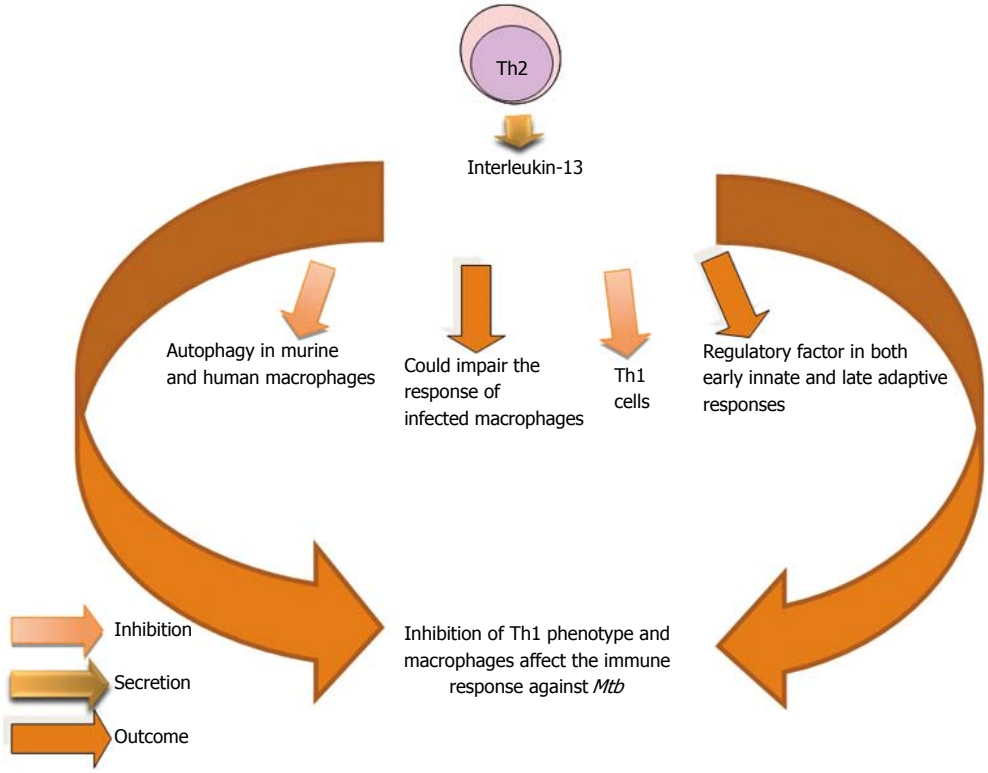

Figure 17 Participation of interleukin-13 in *Mycobacterium tuberculosis* infection. Th1: T helper type 1; Th2: T helper type 2; *Mtb: Mycobacterium tuberculosis*.

Autophagy is a major intracellular pathway for the lysosomal degradation. Therefore, IL-13 could specifically impair the response of infected macrophages[194].

IL-13 released by macrophages infected with virulent strains of *Mtb*[83,85] act in an autocrine manner to inhibit the autophagic process. Treatment of *Mtb*-infected macrophages with either IL-4 or IL-13 promotes the intracellular survival of the bacteria[194] (Figure 17).

IL-17

Generation of human Th17 cells is dependent on IL-23[269,270], IL-1β[269,271,272], TGFβ[270] and IL-6[272]. IL-17A is a cytokine that participates as an immunomodulator in chronic immunological diseases such as: rheumatoid arthritis and inflammatory bowel disease. It can control the pathological mechanisms in the *Mtb* infection through the dysregulating cytokines and chemokines production and promoting granuloma formation. It has been observed that IL-17A significantly enhanced the clearance of intracellular Bacillus Calmette-Guérin (BCG) by macrophages through nitric oxide (NO) -dependent killing mechanism[273].

During the initial stages of infection IL-17 acts on different types of cells and stimulates the secretion of antimicrobial peptides, granulocyte colony-stimulating factor (G-CSF) and cysteine X cysteine (CXC) chemokines. As DCs migrate to the lymph node, both Th1 and Th17 cell are differentiated. Chemokines in the infected lung promote recruitment of protective cells and a mononuclear granuloma is formed, where IL-23 and IL-6 are highly expressed. IL-17-producing cells accumulate in high numbers in the lungs and immunopathological consequences develop[274].

Other investigations appoint that the involvement of Th17 cells remains to be clarified in relation to TB. Researchers demonstrated that *Mtb*-specific Th17 cells are undetectable in peripheral blood and BALs from TB patients[275].

It has been demonstrated that IL-17 plays an important role in the recruitment of neutrophils to the site of inflammation[275-278], including the airways during infection[279,280]. This cytokine is produced by a variety of host cells, including myeloid cells[281], invariant natural killer (iNK) T cells[282], NK cells[283,284], γδ T cells[285-287] and Th17 cells[288]. IL-17 can downregulate IL-10 production and modulate the Th1 response, which has been demonstrated in models immunized with BCG[289]. This vaccination induces Th17 cells that populate the lungs of immunized mice. Th17 cells recruit Th1 cells to the site of infection to restrict mycobacterial growth, upon challenge with *Mtb*[290]. IL-17 can promote tissue damage during *Mtb* infection[274,291] and in the context of other infectious and autoimmune diseases[276,277,292-294]. An increment of IL-17 production is associated with increased neutrophil recruitment and exacerbated lung tissue pathology after repeated BCG vaccinations[295].

B cells can optimize BCG-elicited Th1 immunity by regulating the IL-17/neutrophil response[296]. In human tuberculous pleural effusion (TPE) Th17 cells and regulatory T cells (Tregs) have been found to be increased. Th17 cells were significantly increased in TPE due to local generation and differentiation stimulated

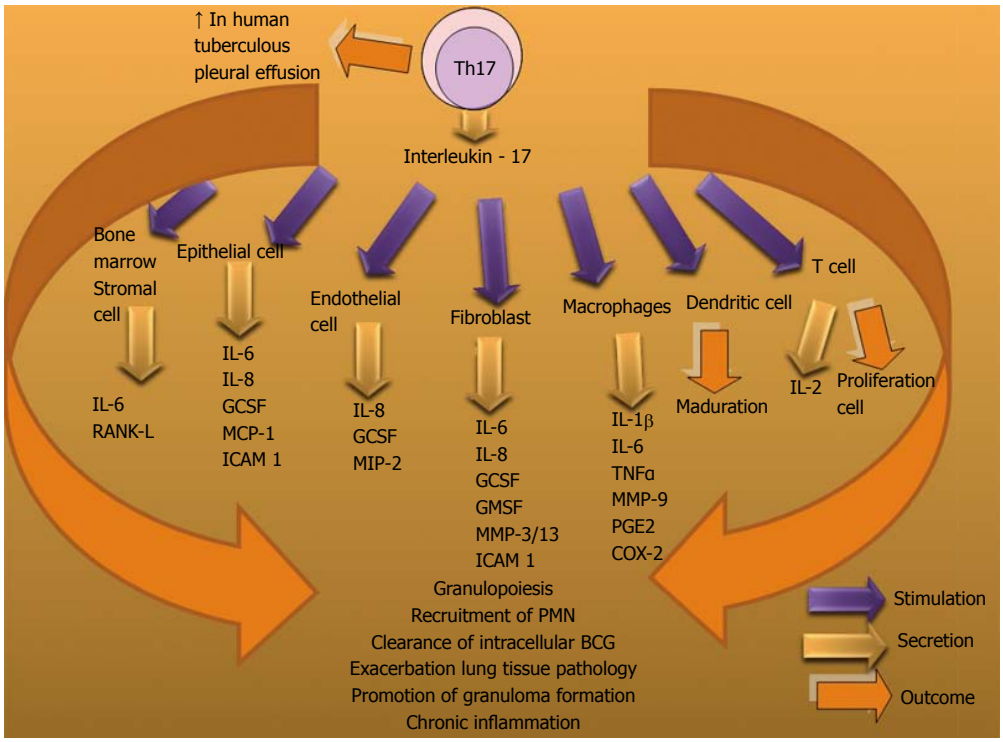

Figure 18 Interleukin-17 in the control of *Mycobacterium tuberculosis* infection. MCP: Monocyte chemoattractant protein; RANK-L: Receptor activator of NF-Kb ligand; MMP-9: Metalloproteinases-9; GCSF: Granulocyte colony stimulating factor; MIP-2: Macrophage inflammatory protein-2; BCG: Bacillus calmette guérin; PGE2: Prostaglandin e-2; COX-2: Cyclooxigenase-2; ICAM 1: Intercellular adhesion molecules-1; IL: Interleukin.

by IL-1β and/or IL-6. $CD39^+$Tregs might participate in the suppression of local immune responses by inhibiting Th17 phenotype[297] (Figure 18).

IL-18

Interleukin- 18 (IL-18) was designed as an IFN-γ-inducing factor, which induces IFN-γ production by splenocytes, hepatic lymphocytes, and type 1 T helper (Th1) cell clones. Its biological actions appear to be similar to those of IL-12[298-300].

Mtb infection in the absence of IL-18 diminishes the Th1 phenotype response. Also, IL-17, chemokines as: CXCL-1 and CXCL-2 cause PMN influx, which exacerbates the immunopathology in IL-18 KO mice. This reveals its immune protective role against *Mtb*[301]. It has been demonstrated that treatment with exogenous IL-18 reduced the bacterial load. This finding does not agree with the studies that have demonstrated that the sizes of the granulomatous lesions in IFN-γ-KO and TNF-α-KO mice infected with *Mtb* were not reduced significantly by recombinant IFN-γ or TNF-α[302].

The inflammatory lesions in IL-18-KO mice were no more severe than those observed in IFN-γ-KO[23,257], TNF-α-KO mice[302] and IL-12-KO[303]. Therefore, IL-18 does not seem to play a role in *Mtb*-induced granuloma formation (Figure 19).

IL-22

IL-22, a member of the IL-10 family, is mainly produced by T and NK cells[304,305]. It is considered to be produced by Th17 cells in an IL-23-dependent manner[306,307] or by a private T cell lineage termed Th22[308,309].

Previously, it had been shown that IL-22 produced by NK cells in humans and $CD4^+$ T cells in macaques could limit *Mtb* growth in macrophages by increasing phagolysosomal fusion. However, IL-22 can play a dual role in tissue homeostasis depending on the cytokine microenvironment where it is induced[310,311].

Study suggests that $NK1.1^+$ cell-derived IL-22 contributes to vaccine-induced protective immunity but not to the primary immune response to *Mtb,* as is the case for IL-17. IL-17 and IL-22 appear to mediate vaccine-induced protective immune responses; however, different mechanisms are involved. IL-17 induces local chemokine production, which leads to optimal priming of T-cells[306], whereas IL-22 inhibits expansion of induced Tregs and enhances antigen-specific T-cell responses, resulting in a reduced bacillary burden after challenge with *Mtb* virulent strains (H37Rv). The mechanisms through which IL-22 inhibits Treg expansion and enhances T-cell responses remain uncertain[310].

IL-22 levels in pericardial fluid correlated positively with MMP-9, an enzyme known to degrade the pulmonary extracellular matrix. Levels of MMP-9 in blood are associated with severity of TB disease[312].

Researchers reported significantly higher IL-22 levels in BAL fluid from patients with pulmonary TB, compared with healthy controls. Also, levels of IL-22 in pleural effusion and pericardial effusions from TB patients were

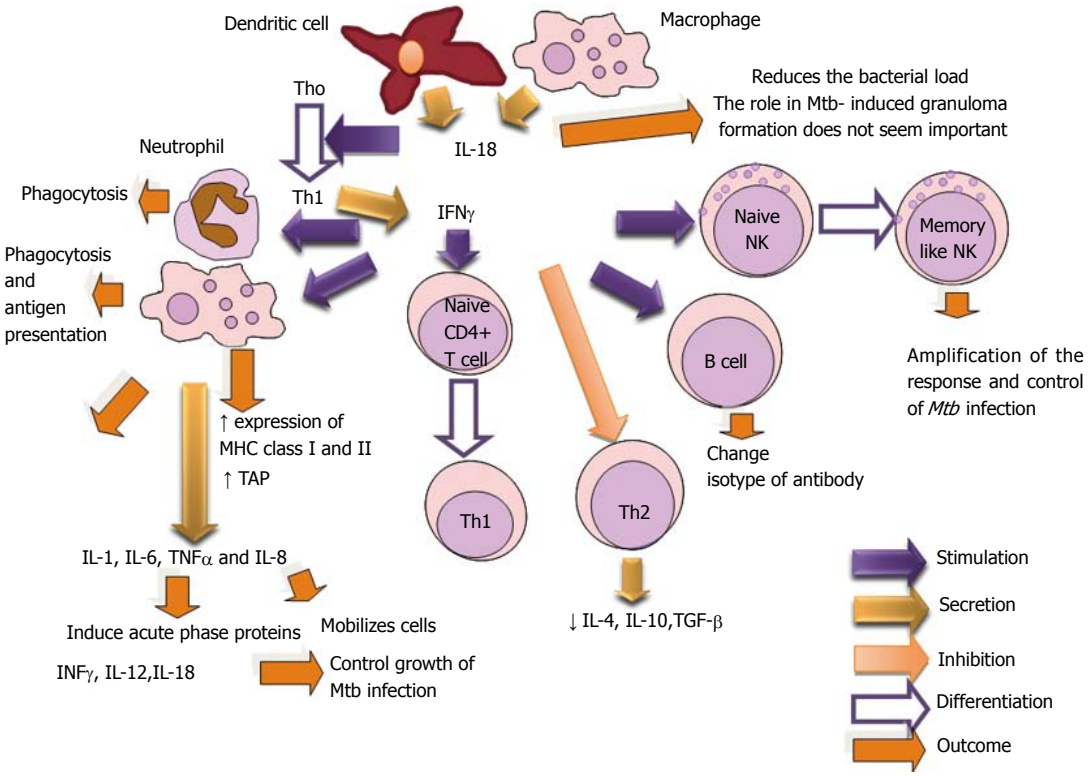

Figure 19 Role of interleukin-18 in *Mycobacterium tuberculosis* infection. TGF-β: Transforming growth factor-beta; NK: Natural killer; TAP: Transporters associated with antigen processing; CD: Clusters of differentiation; IL: Interleukin.

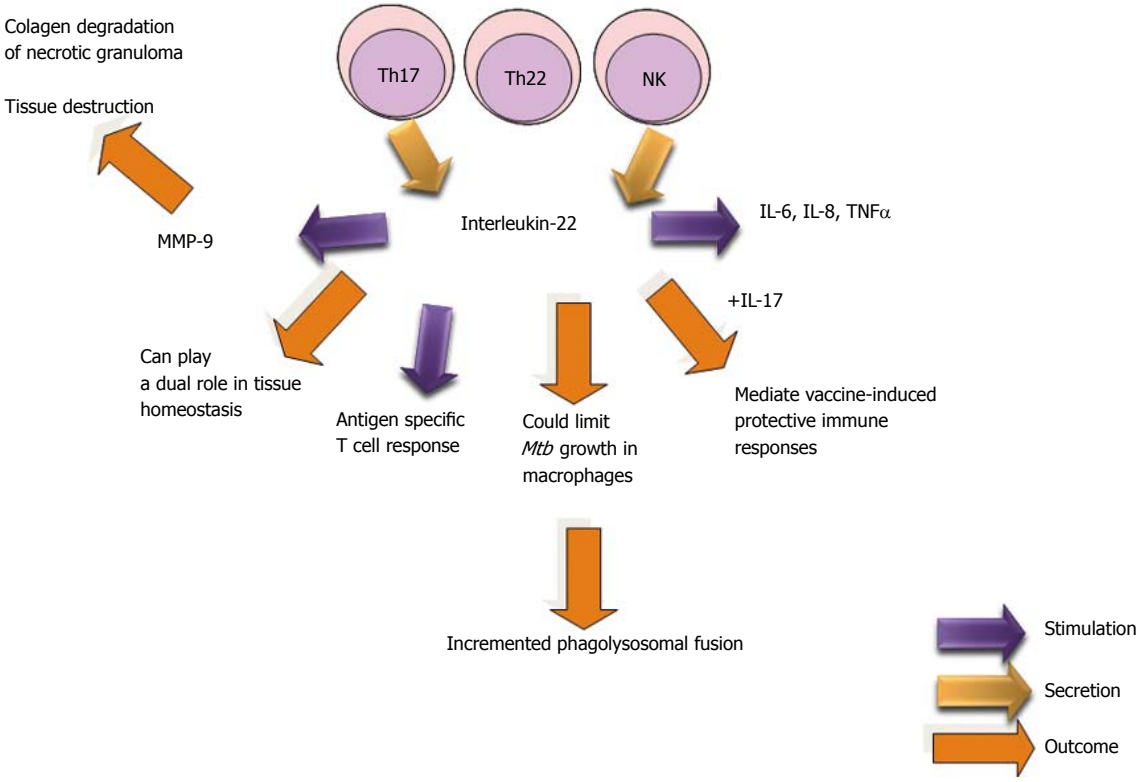

Figure 20 Biological Effects of interleukin-22 in the *Mycobacterium tuberculosis* infection. MMP-9: Metalloproteinases-9; NK: Natural killer; IL: Interleukin.

readily detectable in most[313].

It has been detected IL-22-producing T cells in lung tissue sections and granulomas of *Mtb* infected macaques[314]. However, the treatment of *Mtb* infected

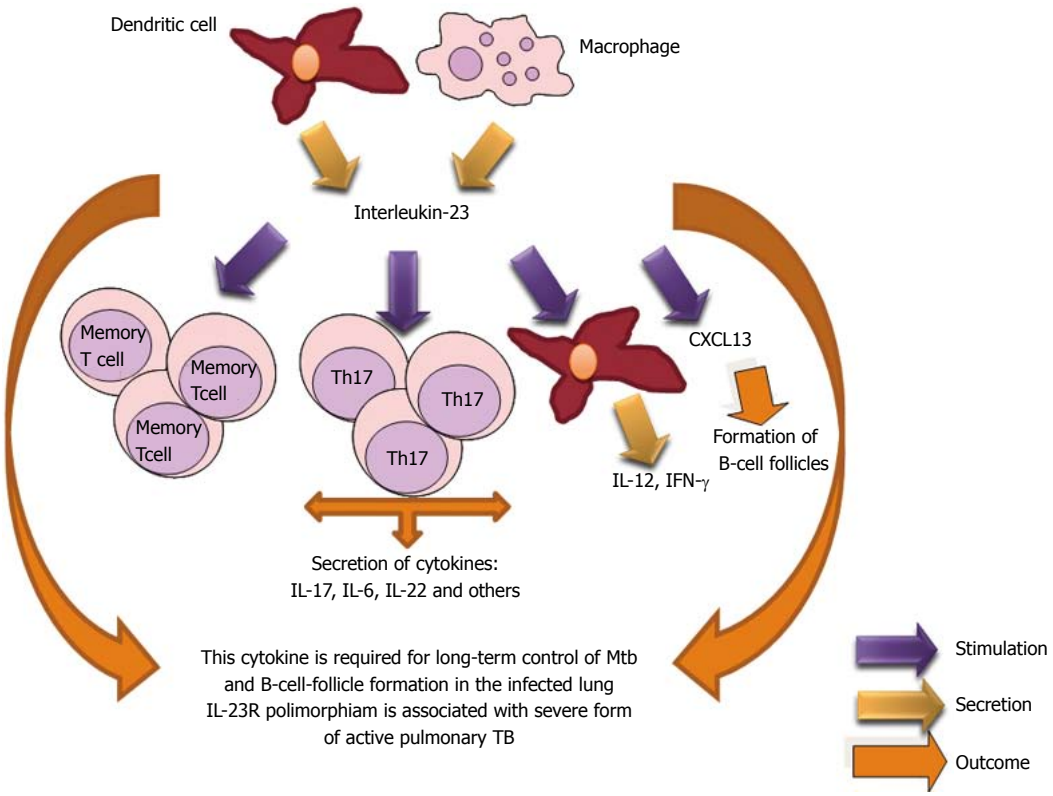

Figure 21 Participation of interleukin-23 in *Mycobacterium tuberculosis* infection. TB: Tuberculosis; IL: Interleukin; IFN-γ: Interferon-γ.

mice with neutralizing anti-IL-22 antibodies did not affect pathology, granuloma formation or bacterial burdens in the lung[315] (Figure 20).

IL-23

IL-23 is a new IL-12 family member. IL-23 mediates its activity through IL-23R. IL-23 is a heterodimeric cytokine composed of a p19 subunit and a p40 subunit. Also, stimulates the proliferation of Th17 cells, a phenotype which produces inflammatory cytokines such as IL-17, TNFα, and IL-6[316,317]. IL-23 is necessary for the expression of IL-17A and IL-22 in the lung. The absence of IL-23 affects the expression of CXCL13 (B cell chemoattractant) within *Mtb*-induced lymphocyte follicles in the lungs and its deficiency is associated with increased T cells around the vessels in the lungs of studied mice[318]. The absence of homeostatic chemokines delays the protective immunity and granuloma formation[319]. Study has demonstrated that the *IL23R* (Arg381Gln) functional polymorphism is associated with an increased risk of development of a severe form of active pulmonary TB. Further studies are necessary[320] (Figure 21).

IL-27

IL-27 is a member of the IL-12 family. IL-27 is an important inhibitory cytokine for the Th17 differentiation and limits inflammation of autoimmune and infectious origin[321-323]. Although the role of this cytokine is still not well understood in TB, two different studies have shown that IL-27R signaling has detrimental effects for the control of *Mtb* in the mouse model[324,325]. IL-27 can serve as a counter-regulatory of cytokines to prevent extensive immunopathology by keeping cellular responses in control. This cytokine can modulate the intensity and duration of many classes of T cell responses[326] (Figure 22).

TGF-β

TGF-β is a key mediator in the immunopathogenesis of TB because it is able to affect quantitative and qualitatively other cytokines, such as IL-1β and TNF-α, and modulate the functions of T lymphocytes and macrophages[327-330]. In pleural tuberculosis, the excessive production of TGF-β is believed to be related to the clinical progression of the disease, particularly in the physiopathology of pleural thickening. TGF-β possesses proinflammatory activity in low concentrations (pleural tuberculosis and healthy contacts of tuberculosis carriers) and anti-inflammatory activity in high concentrations (pulmonary tuberculosis)[329]. It has observed increased levels of TGF-β in the pleural fluid and blood of tuberculosis patients. Although higher TGF-β levels were observed in the pleuropulmonary form, there was no statistical significance when compared to the levels in patients with pleural disease[331]. Pediatric TB is associated with elevated plasma levels of TGF-β, IL-21, and IL-23, which reveal an important role in the disease pathogenesis[332] (Figure 23).

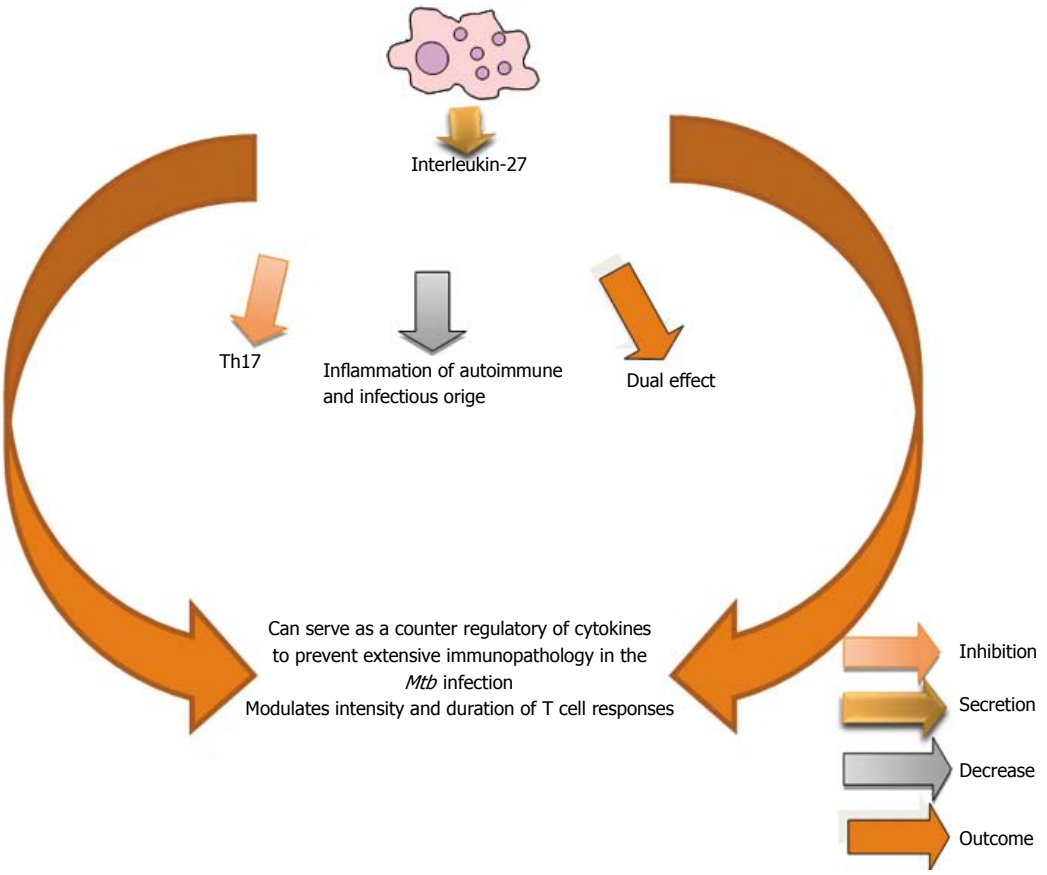

Figure 22 Role of interleukin-27 in *Mycobacterium tuberculosis* infection. Th17: T helper type 17; *Mtb: Mycobacterium tuberculosis*.

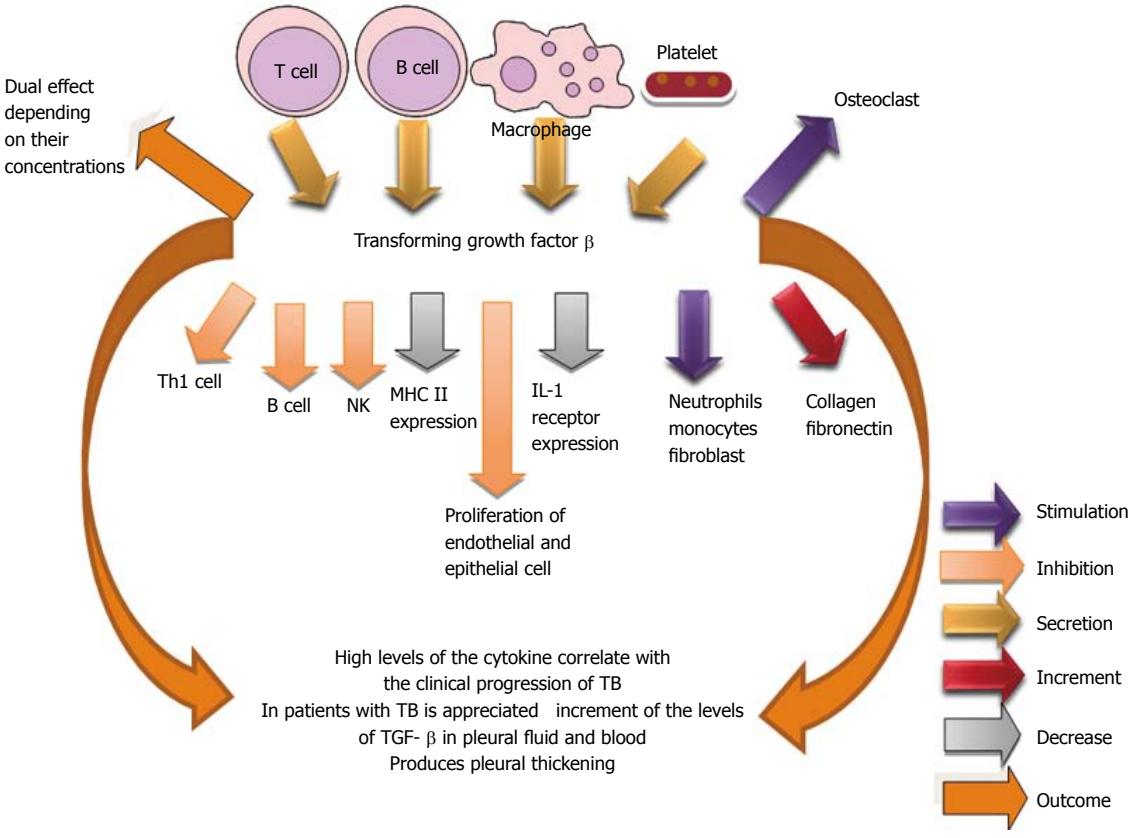

Figure 23 Transforming growth factor-beta in the control of *Mycobacterium tuberculosis* infection. TB: Tuberculosis; Th1: T helper type 1; TGF-β: Transforming growth factor-beta; NK: Natural killer; IL: Interleukin.

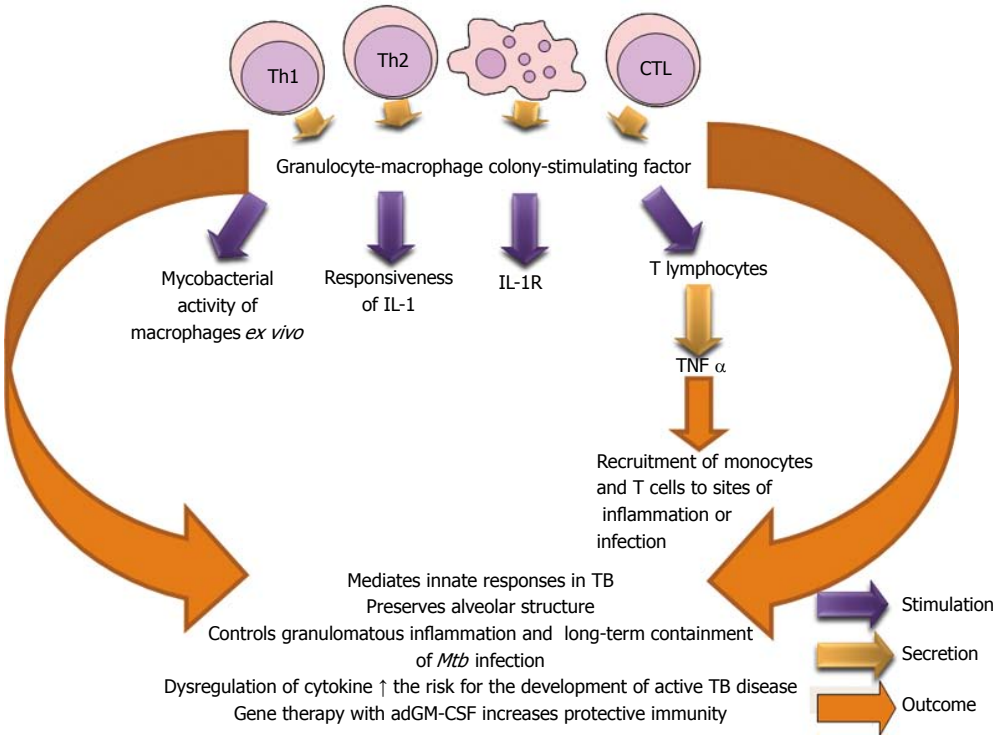

Figure 24 Influence of granulocyte macrophage colony-stimulating factor in the control of *Mycobacterium tuberculosis* infection. GM-CSF: Granulocyte macrophage colony-stimulating factor; IL: Interleukin; *Mtb*: *Mycobacterium tuberculosis*; TB: Tuberculosis; TNF: Tumor necrosis factor.

GM-CSF

GM-CSF is produced by Th1, Th2 and CTL. This cytokine augments the production of granulocytes, macrophages and dendritic cells. GM-CSF and IL-3 stimulate the production of new macrophages when those cytokines act on primary hematopoietic cells of the bone marrow. GM-CSF has a fundamental role in a balanced innate host defense against tuberculosis by its role in preserving the integrity of alveolar epithelial cells and in regulating macrophages and dendritic cells to facilitate containment of virulent mycobacteria in pulmonary granulomas. Prolonged dys-regulation of GM-CSF expression favors the development of pulmonary tuberculosis in immuno-competent individuals[333]. Impaired GM-CSF signaling determines a defective innate activity in alveolar macrophages and allows high susceptibility to lung infections[334].

Invariant natural killer T (iNKT) cells produced GM-CSF in vitro and in vivo in a cluster of differentiation molecule 1 d (CD1d)-dependent manner during *Mtb* infection, and GM-CSF were both necessary and sufficient to control *Mtb* growth. GM-CSF has a potential role in T cell immunity against *Mtb*[335]. GM-CSF and/or TNF-α contributed with the most successful cellular differentiation and appoint that the culture conditions can limit or favor the replication of the bacteria in macrophages[57].

GM-CSF is an important cytokine in the immune protection against Mtb and gene therapy with recombinant adenoviruses encoding granulocyte-macrophage colony-stimulating factor increased protective immunity when administered in a model of progressive disease, and when used to prevent reactivation of latent infection or transmission[336] (Figure 24).

Chemokines

Chemokines belong to a large family of proteins called chemotactic cytokines and have an average molecular mass of 8-14 kDa. They can mediate the constitutive recruitment of leukocytes from the blood into tissues[337,338].

CCL2 (monocyte chemoattractant protein, MCP-1) promotes polarization to Th2 phenotype, resulting in a defective control of Mtb infection. Serum levels have been associated with TB disease activity and treatment response. CCL-3 [macrophage inflammatory protein 1α (MIP-1α)] mobilizes more Th1 than Th2 cells. CCL5 (Regulated upon Activation, Normal T cells Expressed and Secreted, RANTES) is important in the recruitment of Th1 cells to form lymphocyte-enriched granulomas and it has a relevant role in early response of IFNγ producing Tcells. CCL7 (monocyte chemoattractant protein-3, MCP-3) mobilizes phagocytic cells, NK cells and T Lymphocytes. This chemokine has been found elevated in bronchoalveolar lavage fluid and biopsy specimens of subjects with pulmonary tuberculosis. CCL12 (monocyte chemoattractant protein-5, MCP-5) is chemotactic for eosinophils, monocytes, and T and B lymphocytes. CXCL2 (GRO-β) mobilizes neutrophils and fibroblasts. CXCL8 (IL-8) increases the capacity of

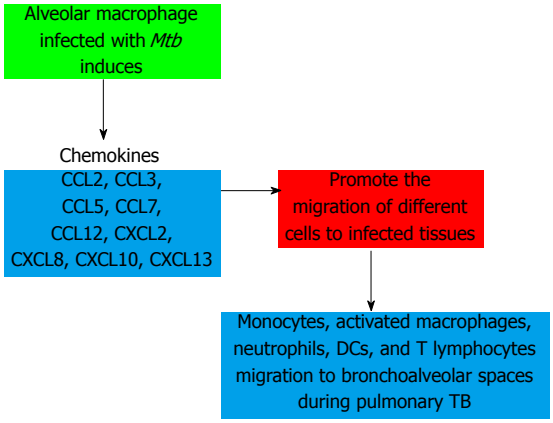

Figure 25 Participation of chemokines in pulmonary tuberculosis. TB: Tuberculosis; DCs: Dendritic cells.

the neutrophil to kill *Mtb* among other functions and CXCL10 (IFN-γ induced protein, IP-10) is a good marker for monitoring of the treatment in adults with active TB[337, 338].

IL-8 (CXCL8) is a strong neutrophil, monocytes and T-cells chemoattractant[339,340]. TB patients presented increased of CXCL8 levels in plasma and bronchoalveolar lavage fluids[341,342] which activates phagocytes as neutrophilis[343]. These cells are found in abundantly in the sputum of TB patients and are persistently recruited to sites of chronic mycobacterial infection[344,345]. Researchers have demonstrated that Mycobacterial infection of alveolar epithelial cells induce the secretion of CXCL8 and IL-6, but not secretion of the monocyte chemotactic CCL2 or pro-inflammatory TNF-α[346].

Study reveals that up-regulation of CCL18 and IL-10 in macrophages by *Mtb* may be involved in the recruitment of naïve Tcells in association with local suppressive immunity against intracellular pathogen[347]. The relationship between mycobacterial antigen-induced IFN-γ and CXCL9 may play a role in determining disease severity in TB[348]. CCL20 attracts immature dendritic cells and down-regulates the characteristic production of reactive oxygen species induced by *Mtb* in monocytes which may affect the activity of the cells. This chemokine inhibits apoptosis mediated by the mycobacteria[349]. CCL22 might be capable of inducing the migration of Tregs to the pleural space of the patients with TPE[350].

It has proposed a model in relation to the formation of granuloma and the participation of chemokines for host defense during *Mtb* infection. Alveolar macrophages uptake *Mtb* and secrete chemokines. Other cells such as epithelial cells and fibroblasts also produce chemoattractant proteins. This chemokine cascade causes an initial recruitment of neutrophils and monocytes. Meanwhile, lung DCs infected by *Mtb* increase the expression of CCR7 and migrate in response to chemokines expressed within lymphoid organs to polarize T cells into phenotypes productors of cytokines. CXCL13 and CCL19 may then mediate correct spatial localization of immune cells to form granulomas and mediate the control of *Mtb*[351] (Figure 25). The complexity of the network of cytokines and chemokines is observed by a recent study which shows that patients with coinfection HIV/*Mtb* have similar pattern of regulating proteins. Anti TB treatment significantly improves the level of pro-inflammatory cytokines (Th1 phenotype) and chemokines but does not restore the immune response in HIV-positive patients[352].

CONCLUSION

The existence of lineages, sublineages, strains and substrains reveal the complexity of *Mtb* and hence differences in the behavior of the immune system and the evolution of the disease. There is evidence suggesting some strains of *Mtb* may result in higher rates of disease progression, treatment failure, and relapse. The presence of persisting immune activation and high frequencies of Treg lymphocytes may reflect immune dysregulation that predisposes individuals to clinical tuberculosis, specifically to extrapulmonary tuberculosis. There is no doubt that immunity to *Mtb* depends on Th1-cell activity (IFN-γ and IL-12 and the production of TNF-α), but Th1 immunity alone is not sufficient to protect the host from *Mtb* infection, development of the disease, or dissemination. CD8+ T and γδ T cells exhibit cytolytic effector functions in the *Mtb* infection which amplifies the response. Studies of B cell immunodeficiency in both humans and mice have questioned whether these lymphocytes impart a protective effect against *Mtb*.

Recently, has been demonstrated that NK cells have the capacity for memory-like responses which permit greater control of the bacterial infection. IFN-γ, TNF-α, IL-12 and IL-17 are important participants in Mycobacterium-induced granuloma formation. Cytokines produced by Th17 phenotype enhance the clearance of intracellular Bacillus Calmette-Guérin (BCG) by macrophages.

IL-27 and IL-10 can serve as counter-regulatory of the cytokines to prevent extensive immunopathology by keeping anti-bacterial cellular response in control. The excessive production of TGF-β is believed to be related to the clinical progression of the disease, particularly, in the physiopathology of pleural thickening. Some chemokines may mediate correct spatial localization of immune cells to form granulomas and mediate the control of *Mtb* and others are also involved in the migration of different cells to infected tissues. As appreciated, the immune system is an extensive network and its final outcome is based in biological actions of cytokines and the participation of the factors aforementioned.

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EDITORIAL

Antimicrobial lipids: Emerging effector molecules of innate host defense

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Abstract

The antimicrobial properties of host-derived derived lipids have become increasingly recognized and evidence is mounting that antimicrobial lipids (AMLs), like antimicrobial peptides, are effector molecules of the innate immune system and are regulated by its conserved pathways. This review, with primary focus on the human body, provides some background on the biochemistry of lipids, summarizes their biological functions, expands on their antimicrobial properties and site-specific composition, presents modes of synergism with antimicrobial peptides, and highlights the more recent reports on the regulation of AML production as well as bacterial resistance mechanisms. Based on extant data a concept of innate epithelial defense is proposed where epithelial cells, in response to microbial products and proinflammatory cytokines and through activation of conserved innate signaling pathways, increase their lipid uptake and up-regulate transcription of enzymes involved in antimicrobial lipid biosynthesis, and induce transcription of antimicrobial peptides as well as cytokines and chemokines. The subsequently secreted antimicrobial peptides and lipids then attack and eliminate the invader, assisted by or in synergism with other antimicrobial molecules delivered by other defense cells that have been recruited to the site of infection, in most of the cases. This review invites

reconsideration of the interpretation of cholesteryl ester accumulation in macrophage lipid droplets in response to infection as a solely proinflammatory event, and proposes a direct antimicrobial role of lipid droplet-associated cholesteryl esters. Finally, for the interested, but new- to- the-field investigator some starting points for the characterization of AMLs are provided. Before it is possible to utilize AMLs for anti-infectious therapeutic and prophylactic approaches, we need to better understand pathogen responses to these lipids and their role in the pathogenesis of chronic infectious disease.

Key words: Atopic dermatitis; Cholesterol; Infectious disease; Cystic fibrosis; Mucosa

Core tip: The antimicrobial properties of host-derived derived lipids have become increasingly recognized. This review develops the concept of antimicrobial lipids (AMLs) as effectors of the innate immune response that work together with antimicrobial peptides to prevent infection, and highlights more recent reports on the regulation of AML production as well as bacterial resistance mechanisms. Furthermore, this review invites reconsideration of the interpretation of cholesteryl ester accumulation in macrophage lipid droplets in response to infection as a solely proinflammatory event, and proposes a direct antimicrobial role of lipid droplet-associated cholesteryl esters.

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INNATE IMMUNITY

Innate immunity is the first line of host defense; it engages pattern recognition receptors as opposed to highly variable antigen specific receptors utilized by the adaptive immune system; its response is preformed or rapidly induced within minutes to hours after pathogen contact; it provides no memory, but is essential for priming the adaptive immune response; and in return it can be augmented by effectors of the adaptive immune response[1,2]. The innate immune response is activated by microbial products and proinflammatory cytokines when general physical and chemical defense mechanisms on body surfaces have failed to eliminate potential intruders. Ligand-binding to surface-expressed and intracellular pattern recognition and cytokine receptors leads to increased output of antimicrobial effector molecules, chemokines, and cytokines to attack the pathogen, recruit, and activate additional immune cells, respectively. The associated signaling pathways are conserved and utilize common central transcription factors including nuclear factor κB and interferon response factors.

Key effector cells of the innate immune response are epithelial cells, granulocytes, monocytes, macrophages, dendritic cells, and natural killer cells. In particular, macrophages and dendritic cells are important for the initiation of the adaptive immune response. In addition, the more recently recognized innate lymphocytes facilitate the cross talk between innate and adaptive immune responses[3]. Key effector molecules with direct antimicrobial action include the complement system, antimicrobial peptides and proteins (AMPs), and, increasingly recognized, antimicrobial lipids (AMLs). This review aims to introduce the concept of lipids as antimicrobial effector molecules in the innate epithelial cell defense. The reader is directed to Thormar and Hilmarsson 2007[4], Drake *et al*[5], 2008, and Thormar[6] 2012 for more extensive previous reviews on antimicrobial properties of lipids.

BIOCHEMICAL CHARACTERISTICS OF LIPIDS

Lipids are a widely heterogeneous group of molecules that share hydrophobic or mixed hydrophobic/hydrophilic properties. They are composed of hydrocarbon chains to which additional functional groups are linked which affects the degree of hydrophobicity. The major lipid classes are: fatty acids, tri-, di- and mono-acylglycerols consisting of the alcohol glycerol and fatty acid chains, cholesterol and cholesteryl esters, phospholipids and sphingolipids. Mostly, fatty acids, acyl chains with a carboxy group, are incorporated into more complex lipids. For example, sphingolipids like sphingosines consist of a fatty acid residue linked to an amino alcohol and cholesteryl esters are formed through esterification of a fatty acid to cholesterol. Phospholipids typically consist of a glycerol with two fatty acid residues attached, a phosphate group and varying additional groups such as choline, an alcohol, or amines. Phosphosphingolipids such as sphingomyelin use sphingosine instead of the diglyceride. Free fatty acids (FFA) are less abundant in the body, and among them palmitic, stearic, oleic, linoleic (the latter three differing in the number of double bonds) and docosahexaenoic acid are possibly the most important in the current context. Linoleic acid and its metabolite arachidonic acid are essential and cannot be synthesized by humans. Otherwise, our body generates all other fatty acids by two-carbon chain additions to acetyl-coenzyme A (CoA). For more detailed information on their classification refer to Fahy *et al*[7] and Christie and Xianlin[8].

Though lipid biosynthesis is quantitatively most active in hepatocytes and adipose tissue, every nucleated cell is capable of it. Figure 1 gives an overview of lipid biosynthesis as it relates to the production of AMLs and earmarks the enzymes for which evidence of regulation by innate immune pathways is available.

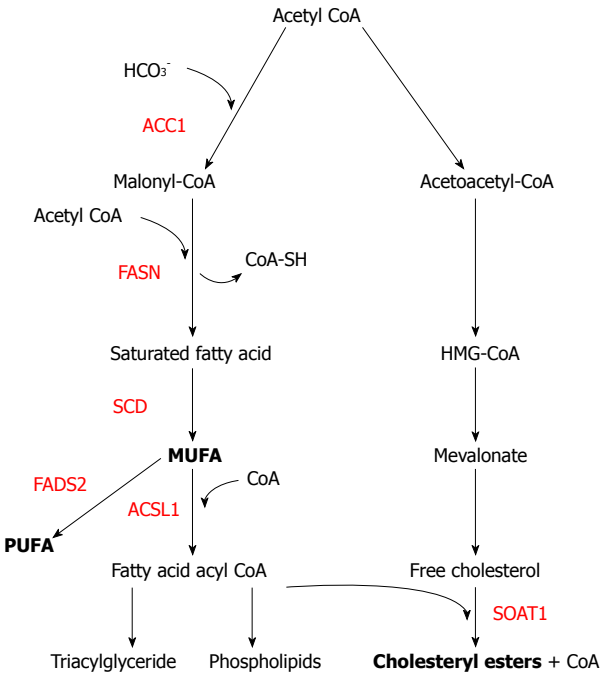

Figure 1 Simplified lipid biosynthesis pathway highlighting the lipids and the enzymes with a putative role in innate immunity. Lipid classes with documented antibacterial activity are in bold, key enzymes that may be induced in response to infection and inflammation (*homo sapiens* nomenclature) are in red. MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; ACC1: Acetyl-CoA carboxylase 1; FASN: Fatty acid synthase; SCD: Stearoyl-CoA desaturase-1; ACSL1: Acyl-CoA synthetase long-chain family member 1; FADS2: Fatty acid desaturase 2; SOAT1: Sterol O-acyltransferase 1 (SOAT1, also known as acyl-Coenzyme A: Cholesterol acyltransferase 1 or ACAT 1); HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA.

The initial and committed step in the fatty acid synthesis pathway is mediated by acetyl-CoA carboxylase 1 that catalyzes the addition of CO_2 to the methyl group of acetyl CoA generating malonyl-CoA. Malonyl-CoA serves as the donor of two carbon acetyl groups during each round of the fatty acid synthesis reaction cycle. Fatty acid synthase is a multifunctional enzyme that catalyzes seven different reactions where two carbon units from malonyl-CoA are linked together ultimately resulting in the formation of saturated fatty acids. Terminal desaturases then generate unsaturated fatty acids. Stearoyl-CoA desaturase also known as delta-9-desaturase catalyzes the synthesis of monounsaturated fatty acids (MUFAs). Biosynthesis of MUFAs occurs through the introduction of the first cis double bond in the Δ9 position between carbons 9 and 10. Fatty acid desaturase 2, encoded by FADS2 and also known as delta-6 desaturase, is required for the synthesis of polyunsaturated fatty acids (PUFAs). FADS2 is classified as a front-end desaturase because it introduces a double bond between the pre-existing double bond and the carboxyl end of the fatty acid. Long-chain-fatty-acid-CoA ligase 1 is encoded by ACSL1 and converts free long-chain fatty acids into fatty acyl-CoA esters. Acyl-CoA synthetases activate free long-chain fatty acids by converting them into fatty acyl-CoA esters. Fatty acyl-CoA esters are substrates for multiple fatty acid metabolic pathways, including mitochondrial β-oxidation and phospholipid and triacylglycerol synthesis. Sterol O-acyltransferase 1 (SOAT1, also known as acyl-Coenzyme A: cholesterol acyltransferase 1 or cholesterol acyltransferase 1), catalyzes the esterification of fatty acids to cholesterol. An ester bond is formed between the carboxylate group of a fatty acid and the hydroxyl group of cholesterol. *De novo* synthesis of free cholesterol *via* the mevalonate pathway also begins with acetyl CoA. Acetyl-CoA undergoes condensation with another acetyl-CoA subunit *via* acetyl-CoA transferase to form acetoacetyl-CoA. Acetyl-CoA condenses with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is reduced to mevalonate with consumption of NADPH, and after sequential reactions producing the intermediates mevalonate-P, isopentenyl-PP, farnesyl-PP, squalene, lanosterol, and 7-dehydrocholesterol, free cholesterol has been generated.

BIOLOGICAL FUNCTIONS OF LIPIDS

Lipids are used as a form of energy storage, are precursors for steroid hormones[9], and have important structural functions. Cell membranes are composed of a phospholipid bilayer and transmembrane receptor signaling is dependent on the specific lipid composition of the cell membrane in the vicinity of these receptors. These specialized regions are referred to as lipid rafts and caveolae[10-12]. There are substantial differences in the phospholipid composition of bacterial and mammalian cell membranes, likely contributing to the preferential action of host defense molecules against bacterial targets[13-15]. Furthermore, lipids liberated from cellular membranes have been found to be strong modulators of inflammation. Initially, they were identified as strong proinflammatory second messengers such as prostaglandins and leukotrienes which are synthesized from arachidonic acid. However, in the last decade an important down regulatory role of membrane-derived lipids has been discovered. These inflammation resolving lipids are derivatives of the essential omega-6 and omega-3 PUFAs and include resolvins (coined after their inflammation resolving function), lipoxins, protectins and maresins[16-18]. Moreover, there is new evidence that lipids may also trigger increased antimicrobial peptide production as shown for the sphingolipid S1P which increased CAMP production[19], or for sebum FFA which induced beta-defensin production[18]. However, lipids can also exert direct antimicrobial activity, which is not only supported by *in vitro* testing but also by the association of some infectious diseases with defects in lipid metabolism.

CLINICAL CORRELATIONS BETWEEN LIPID ALTERATIONS AND INFECTIONS

Several chronic infectious diseases are associated with altered lipid composition in skin and in the airways.

For example, Arikawa *et al*[20] reported reduced sphingosine levels in keratinocytes in patients with atopic dermatitis and recurrent *Staphylococcus aureus* (*S. aureus*) skin infections. In the stratum corneum of lamellar ichthyosis patients who are at higher risk of contracting chronic dermatophytosis[21], the amount of FFA is reduced and the ceramide profile is altered[22,23]. In cystic fibrosis, patients suffer from chronic lung infections with *S. aureus*, *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, and, most importantly, *Pseudomonas aeruginosa*[24-27]. In these patients, altered fatty acid levels including reduced levels of docosahexaenoic acid[28] have been described and docosahexaenoic acid supplementation improved the clinical status in some studies[29]. Other lipid anomalies in cystic fibrosis are altered cholesterol homeostasis[30], and elevated cholesteryl ester concentrations in tracheobronchial secretions[31]. We have found an increased cholesteryl ester representation in the lipid content of bronchoalveolar lavages obtained from pediatric cystic fibrosis patients[32]. Furthermore, elevated cholesteryl linoleate levels were found in sinus washes in chronic rhinosinusitis[33].

BODY SITES AND FLUIDS WITH AMLS

Lipids have been well characterized in all body surfaces and tissues whereby extraction and identification method influences the outcome and caution should be applied when comparing results from different studies. Recognition of the antimicrobial activity of certain lipids and improved analytical instrumentation have invited additional surveys many of which are compiled in Thormar[6] 2011. Analysis of the lipid composition of the intestinal tract is complicated by nutritional lipids and lipids synthesized by the normal endemic microbiota and thus, is not considered in the present review.

Breast milk and vernix caseosa

Breast milk was one of the first human body fluids investigated for its lipid content. Thormar *et al*[34] reported in 1987 that FFA and monoglycerides in milk exhibit antiviral activity. It appears that milk lipases release the bioactive lipids from more complex lipids. This work was subsequently extended to include activity against various bacteria and protozoa[35]. Unique to the newborn is vernix caseosa, the waxy coat formed during the last trimester of pregnancy that covers the new born infant. This lipid-rich film is primarily derived from the stratum corneum and sebaceous glands of the fetal skin. Ten percent of its content is represented by lipids, with a relative abundance of nonpolar species such as wax esters/sterol esters/squalene, and triacylglycerol. Other vernix caseosa lipids include FFA, fatty alcohols, cholesterol, diacylglycerol, monoacylglycerol, and phospholipids[36-39]. Antibacterial activity of total lipid extract was observed against the test strain *Bacillus megaterium* and was attributed to FFA.

Skin

Skin lipids (sebum) are from secretions by sebaceous glands and the stratum corneum, their composition is in part further shaped by the metabolic activities of the normal microbiota[4,40] and the exogenous application of lotions and cosmetics. Employing a combined LC/MS approach Camera *et al*[41] identified 95 triacylglycerols, 25 diacylglycerols, numerous wax esters and squalenes, a total of 9 cholesterol esters, and more than 48 FFA in sebum. Antimicrobial activity has been attributed to fatty alcohols, monoglycerides, sphingolipids including D-sphingosine, phospholipids, and in particular FFA such as sapienic acid and lauric acid[42,43].

Meibum

Very long chain wax esters and fatty acids have been of identified in meibum, the lipid rich component of tears[44]. Lipids in tear fluid reach micromolar concentrations and the most abundant species are phosphatidylcholine and phosphatidylethanolamine. Additional lipid classes are triglycerides, sphingosine and ceramides, as well as cholesteryl esters[45]. While a lubricant function has been primarily attributed to tear lipids, a recent study suggested growth inhibitory activity of whole tear lipid extracts against several Gram-positive and Gram-negative bacteria[46].

Oral mucosa

Sphingosine, sapienic and lauric acid have also been identified as key antimicrobial fatty acids in the oral mucosa[47]. Brasser *et al*[48] analyzed salvia from healthy adults and identified FFA, cholesterol, cholesterol esters, triglycerides, wax esters, and squalene. The neutral lipid concentration was determined to be in the low μg/mL range. Overall, FFA, triglycerides, and cholesteryl esters were the most abundant lipids in saliva.

Airways

In the airway lumen, surfactant is the main lipid source ascending from the alveolar space, its primary site of production, to the upper airways, where some local production also occurs[49]. Phospholipids comprise the majority of the lipids in surfactant, a lipoprotein complex[50], and are thought to mainly contribute to reducing lung surface tension and participate in a downregulation of immune responses. The antimicrobial properties of surfactant have been mainly attributed to surfactant proteins SP-A and SP-D[51,52]. Nasal fluid is rich in lipids with all major classes represented, namely FFA, phospholipids, triglycerides, cholesterol, and cholesteryl esters, and their origin can be at least in part attributed to epithelial cell secretions[53]. Selective removal of the non-polar portion of lipids resulted in a decreased inherent antibacterial activity against *P. aeruginosa* that was restored after supplementation with the extracted lipids. This suggests that lipids in nasal fluid contribute to the innate antimicrobial defense in the airways[53].

Urogenital tract

Information on the lipid composition of fluids of the urogenital tract is scarce. Urine contains predominantly phospholipids including glycerophospholipids, phosphatidylcholine, phosphatidyl serine and sphingomyelin, as well as triglycerides, but cholesterol and cholesteryl esters are also present[54,55]. Semen lipids include sphingomyelin, glycerophospholipids, and cholesterol[56,57]. A very recent metabolomics study on bacterial vaginosis suggested elevated eicosanoid levels in affected women but this study was designed to identify differentially represented metabolites in diseases patients and did not aim to provide a baseline lipid profile of healthy women[58]. To the best of our knowledge, information on the antimicrobial activity of lipids of the urogenital tract is not available.

SPECTRUM OF ACTIVITY OF AMLS

Among human lipids, fatty acids are the best characterized as antimicrobial agents, and their spectrum of activity as a whole is broad and spans from bacteria and viruses to fungi and protozoa[6]. Other human lipids with antimicrobial properties include sphingoid bases[43], that are active against Gram positive and Gram negative bacteria. Cholesteryl esters have long been thought to serve only as a storage and transport form for either cholesterol or FFA. However, cholesteryl linoleate and cholesteryl arachidonate, when formulated in liposomes, demonstrated growth inhibitory activity against several Gram positive and Gram negative bacteria[53].

MECHANISMS OF ANTIMICROBIAL ACTION

Influenced by the three dimensional shape and saturation status of the acyl chains AMLs exert their action in different ways. These include disruption caused by interference with the cell membrane with ensuing permeability changes or interference with the activity of membrane bound enzyme complexes and events following lipid peroxidation with radical formation. FFA have been substantially investigated in this respect, and a detailed review on this subject has been authored by Desbois and Smith[59]. More recent studies describe rapid membrane depolarization in *S. aureus* treated with palmitoleate as well as when treated with glycerol ethers, sphingosine, and acyl-amines[60]. As demonstrated by scanning electron microscopy, meibomian lipids from tears cause major structural damage including distortion, loss or regular cell shape, and cell lysis in *S. aureus, P. aeruginosa,* and *Serratia marcescens*[46].

The more pronounced antimicrobial activity of unsaturated FFA compared to their saturated counterparts[61] may be at least in part attributed to lipid peroxidation. Spontaneous generation of a lipid radical at the unsaturated bond leads, under consumption of molecular oxygen, to the production of a lipid peroxyl radical that can react with nearby fatty acids leading to a lipid peroxidation chain reaction. Eventually, these radicals covalently modify adjacent macromolecules[62].

In addition, anti-adhesive effects of lipids have been reported. Milk fat globules from bovine and goat milk reduced attachment of *Salmonella* Enteritidis to HT-29 human adenocarcinoma cells and subsequent internalization[63]. Another more recently described effect of AMLs is inhibition of biofilm production. For example the milk monoglyceride monolaurin (also called lauricidin[64]) inhibits biofilm mass produced by Gram positive bacteria including *Streptococcus mutans* and *S. aureus*[65,66].

SYNERGISM WITH AMPS

Antimicrobial peptides are characterized by an amphipathic structure with cationic and hydrophobic domains and are typically less than 10 kDa in size. Antimicrobial proteins have similar amphipathic domains but are larger and typically consist of additional regions with unique functions, such as lysozyme that hydrolyzes peptidoglycan and lactoferrin that binds iron. AMPs share many of the mechanisms described for AMLs, in particular membrane disruption, and there are several studies documenting synergist activities between these two classes of antimicrobials. Tollin *et al*[38] reported synergistic activity between vernix caseosa lipids and the antimicrobial peptide LL37 whereby this effect was attributed to FFA in vernix. We found synergistic effects between nasal fluid lipid extracts and the antimicrobial peptide human neutrophil peptide HNP1[53], and between the free fatty acid docosahexaenoic acid and lysozyme[67]. The latter study demonstrated that in the presence of lysozyme, docosahexaenoic acid accumulates in the bacterial cell membrane. Nakatsuji *et al*[68] demonstrated synergistic effects between the free fatty acid lauric acid and the antimicrobial peptide HBD2 against *Propionibacterium acnes.* This study also showed that several sebum FFA up-regulate antimicrobial peptide production in sebocytes.

A different type of protein-lipid synergism has been described for human α-lactalbumin made lethal to tumor cells (HAMLET) from human milk, primarily known for its anti-tumor effects[69]. When complexed with oleic acid HAMLET exerts bactericidal effects against *S. pneumoniae via* calcium dependent membrane depolarization[70,71]. Furthermore, acetylation of cationic peptides has been shown to impart antimicrobial activity or increase their antimicrobial activity[72].

REGULATION OF AML PRODUCTION

Reports on lipid profile changes in sepsis[73,74] have suggested that AML production may be regulated in the context of infection that would involve TLR and other pattern recognition receptor signaling and signaling induced by proinflammatory cytokines like IL1β. Important evidence

for the regulation of AMLs by conserved pathways of innate immunity was provided by Georgel *et al*[75] investigating the regulation of stearoyl-CoA desaturase gene expression (*scd1* in mice and *scd* in humans), a rate limiting enzyme for the synthesis of monosaturated fatty acids. They found that the *scd1* gene has numerous NFκB elements in its promoter region and is strongly and specifically induced by TLR2 signaling and that *scd* expression is also induced by TLR2 signaling in a human sebocyte cell line. Furthermore, *scd1*$^{-/-}$ mice developed chronic skin infections.

Using a contrary approach, Wang *et al*[76] have recently shown that overexpression of fatty acid desaturases increases resistance to infection in zebrafish. Other findings that suggest that lipids are regulated by infection and inflammation include the activation of genes important for lipid synthesis in caseation of human tuberculosis granuloma[77].

SOAT1 is essential for cholesteryl ester synthesis and we have shown that non-polar lipids overall and specifically cholesteryl linoleate are elevated in sinus washes obtained from patients with chronic rhinosinusitis[33]. This data suggested an up-regulation of SOAT1 in the context of inflammation which was corroborated by a subsequent study showing increased SOAT1 mRNA expression in sinus mucosa of patients with chronic rhinosinusitis[78]. In addition, cholesteryl esters were increased within the lipid fraction and their concentrations correlated with human neutrophil peptides HNP1-3, markers of inflammation, in bronchoalveolar lavage collected from pediatric cystic fibrosis patients[32]. Direct evidence for the regulation of SOAT1 by inflammation was recently provided by Yin *et al*[79], who showed that oxLDL activates TLR4 and induces the expression of SOAT1 (referred to as ACAT-1) *via* MyD88 and NFκB. Thus, there is clinical and experimental evidence that *in vivo* cholesteryl ester biosynthesis is regulated by inflammation and infection. Additional data supporting the regulation of AMLs by TLR ligands and immunomodulatory cytokines can be found in the NCBI Gene Expression Omnibus (GEO Profiles) data base. Table 1 lists genes involved in lipid metabolism and transport which are regulated by TLR ligands and modulators of the immune system.

Other investigations propose that cholesterol and cholesteryl ester accumulation in response to inflammatory cytokines and infection serve perpetuation of inflammation. For example, Pessolano *et al*[80] described that IL1β increased cholesteryl ester accumulation in smooth muscle cells as part of cholesterol trafficking in atherosclerosis. Similarly, Tall and Yvan-Charvet[81] highlight the proinflammatory effects of increased cholesterol uptake through TLR signaling and inflammasome activation in macrophages. However, considering the direct antimicrobial activity of cholesteryl esters these studies could be revisited to investigate changes in the antimicrobial responses.

TRANSPORTERS OF AMLS

Bearing in mind the hydrophobic nature of AMLs and the aqueous milieu in body fluids, proteins with both hydrophilic domains and hydrophobic pockets likely serve as carriers. Albumin and fatty acid binding proteins are well established carriers for fatty acids. Sterol carrier protein 2 and cholesteryl ester transfer protein assume this role for cholesterol and cholesteryl esters, respectively[82]. In addition, in the airways, the highly hydrophobic protein short palate lung epithelial clone protein 1 binds certain phospholipids and sphingolipids[83,84] and may possibly also function as a cholesteryl ester carrier. However, much research is still needed to dissect the focused delivery of AMLs to the microbial target.

BACTERIAL MECHANISMS THAT MANIPULATE HOST-DERIVED LIPIDS

Host defense mechanisms are continuously challenged by microbial resistance factors and it would be surprising if successful pathogens do not have counter strategies that inactivate AMLs. Both, *S. aureus* and *S. saprophyticus* express a cell wall associated surface protein, SsaF and SssF, respectively, that mediates resistance to the free fatty acid linoleic acid[85,86]. Furthermore, cell wall teichoic acids of *S. aureus* confer resistance to fatty acids from skin sebaceous glands[87].

At this time it is still speculative whether a cholesterol esterase produced by *P. aeruginosa*[88] may represent an additional virulence factor aiding in the inactivation of host-derived antimicrobial cholesteryl esters. Of interest is the recent finding of Cadieux *et al*[89] who identified a lipase in a hypervirulent community-associated methicillin-resistant *S. aureus* strain USA300 that hydrolyzes triglycerides and liberates the free fatty acid linoleic acid with growth inhibitory activity against *S. aureus.* It is possible that the liberation of antibacterial linoleic acid is primarily targeted against other bacteria thereby conferring growth advantage to *S. aureus*. Such a mechanism has been proposed for *Salmonella* where the bacteria induce the production of antimicrobial proteins in the intestine that in turn altered the normal microbiota facilitating infection with the pathogen[90].

Successful pathogens subvert host defense mechanisms that normally control infection. Thus, the ability of *Mycobacterium tuberculosis*, *M. leprae* and other intracellular pathogens to import lipids from the cholesteryl ester-rich lipid droplets that they induce in their host cell[91,92] may be an example for subversion of antimicrobial cholesteryl ester accumulation as part of the innate defense.

AMLS AS EFFECTOR MOLECULES OF EPITHELIAL INNATE DEFENSE

Based on the evidence laid out above, we propose that AMLs take part in the innate epithelial defense controlled by regulatory pathways like antimicrobial proteins and functioning in synergism with AMPs (Figure

Table 1 Genes involved in lipid metabolism and transport regulated by innate immune pathways

Role	Gene name	Encoded protein	Function of the encoded protein	Cellular source	Regulators
Biosynthesis	*acc1*	Acetyl-CoA carboxylase 1	Catalyzes the rate limiting irreversible carboxylation of acetyl-CoA to produce malonyl-CoA	Hepatic tissue[1]	LPS *via* sterol regulatory element-binding protein-1c
	acsl1	Long-chain fatty-acid-coenzyme A ligase	Converts free long-chain fatty acids into fatty acyl-CoA esters	Mp, DC, EN, Mo	LPS, IFN-γ, TNF-α, IL22, Mtb-derived lipopeptide
	elov	Elongation of long chain fatty acids	Possibly implicated in tissue-specific synthesis of very long chain fatty acids and sphingolipids[3]	Mp, DC, CD34+, TE,B, F, EN	LPS, Zy, Schi, IL1, IFN-β, IFN-γ, IL10, TGF-β
	fad	Fatty acid desaturase	Catalyzes biosynthesis of highly unsaturated fatty acids. FADS2 catalyzes production of the mono-unsaturated fatty acid sapienate, the most abundant fatty acid in sebum	Mp,DC, CD34+, TE, B, EN	LPS, Zy, Schi, IL1, IFN-γ, IL10, TGF-β
	fasn	Fatty acid synthase	Catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH	Mp, DC, CD34+, TE, F, EN	LPS, Zy, Schi, IL1, IFN-γ, TGF-β
	lcat	Lecithin cholesterol acyltransferase[2]	Esterifies free cholesterol transported in plasma lipoproteins. Activated by apolipoprotein A-I	Mp, DC, CD8+ DC, B, F	LPS, Schi, IFN-β, IFN-γ, *Yersinia* + IFN-γ, Vit D3 + IFN-γ, IL10
	lipA	Lipase A[3]	Intracellular hydrolysis of internalized cholesteryl esters and triglycerides. Activation of endogenous cellular cholesteryl ester formation	Mo, Mp, DC, TE, EN, K, BrE, L, Mg	TLR agonists, IL1, Type Ⅰ and Ⅱ IFNs, γ, Diff/Polar
	scd[4]	Stearoyl-CoA desaturase	Catalyzes the desaturation of very long chain acyl-CoAs	Mo, Mp, L, CD8+ DC, TE, F, EN, K, BrE, Mg	LPS, Zy, TLR agonist, IL1, Type Ⅰ and Ⅱ IFNs, *Yersinia* + IFN-γ, Vit D3 + IFN-γ, Diff/Polar
	soat1[5]	Sterol o-acyltransferase[3]	Catalyzes the formation of fatty acid-cholesterol esters	Mo, Mp, DC, TE, EN, L, Mg	TLR agonists, Type Ⅰ and Ⅱ IFNs, IL1, Diff/Polar
Transport	*cetp*	Cholesteryl ester transfer protein[3]	Involved in the transfer of insoluble cholesteryl esters in the reverse transport of cholesterol	Mo, Mp, DC, TE, EN, K, L, Mg	TLR agonists, IL1,Type Ⅰ and Ⅱ IFNs, Diff/Polar
	fabp	Fatty acid binding proteins	Intracellular lipid transport	Mp, DC, CD34+, TE, B, F, EN	LPS, Zy, Schi, IL1, Type Ⅰ and Ⅱ IFNs, IL10, TGF-β
	ffar	Free fatty acid receptor	Receptor for short chain fatty acids (FFAR2) and medium to long fatty acids (FFAR1). FFAR2 is expressed at relatively high levels in peripheral blood leukocytes	Mp, DC, CD34+,TE, EN	LPS, Zy, Schi, IL1, IFN-γ, TGF-β
	slc27A	Solute carrier family 27	Translocation of long-chain fatty acids across the plasma membrane. Some involved in bile acid synthesis	Mp, DC, CD34+, TE, B, F, EN	LPS, Zy, Schi, IL1, IFN-γ, IFN-β, IL10, TGF-β

[1]Chen *et al*, J Pineal Res 2011 Nov; 51: 416-25 DOI: 10.1111/j.1600-079X.2011.00905.x; [2]Profiles for mouse only; [3]Profiles for human only; [4]*scd1* in mice; [5]Also known as *acat1* (acyl-Coenzyme A: Cholesterol acyltransferase 1). Data were extracted from NCBI Gene Expression Omnibus (GEO Profiles) and Swiss-Prot (http://www.uniprot.org/). Unless specified otherwise entries were for both mouse and human species. Mo: Monocytes; Mp: Macrophages; DC: Dendritic cells; TE: Thyroid epithelial cells; EN: Endothelial cells; L: Lung epithelial cells; Mg: Microglia; F: Fibroblasts; K: Keratinocytes; BrE: Bronchial epithelial cells; B: B-cells; Dex: Dexamethasone; Diff/Polar: Differentiation and polarization; IFN: Interferon; IL: Interleukin; LPS: Lipopolysaccharide; TGF-β: Transforming growth factor β; TNF-α: Tumor necrosis factor α; VitD3: Vitamin D3; Zy: Zymogen; Schi: *Schistosoma* antigen; Mtb: *Mycobacterium tuberculosis*.

2). Following activation of pattern recognition receptors and cytokine receptors, epithelial cells upsurge the uptake of cholesterol and fatty acids, increase the expression of antimicrobial peptides and enzymes for lipid biosynthesis, scale up the production and secretion of AMLs and antimicrobial peptides, and, combined with antimicrobial effectors from other sources such as macrophages, lead to membrane damage and other disrupting effects on the invading pathogen.

FUTURE DIRECTIONS

The recognition that host-derived lipids form part of the innate antimicrobial defense leads to new questions including the following: What are other microbial targets beyond bacteria and viruses? How are AMLs delivered to pathogens? Do carrier proteins assume this task or do exosomes serve this purpose? Can AMLs be incorporated in novel drug design? Is resistance to AMLs a pathogenicity factor that could be targeted in the management of infectious diseases? Are certain chronic and recurrent infectious diseases linked to defective AML production and/or delivery? Can the lipid mediated arm of host defense be integrated in novel vaccine strategies?

HOW TO WORK WITH LIPIDS?

Commercial tools to study AMLs are relatively under-developed compared to the extensive repertoire for proteomics and genomics. An essential technique for qualitative analysis and the ability to assess a

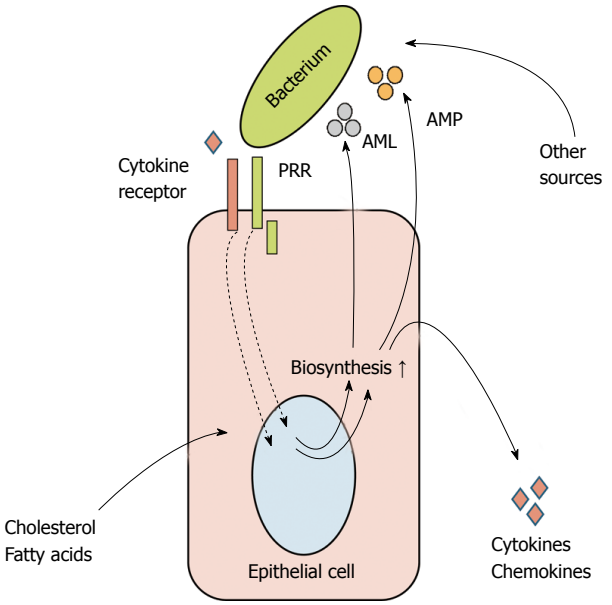

Figure 2 Working model of epithelial cell mediated innate defense. In response to microbial products and cytokines epithelial cells increase the production and secretion of antimicrobial lipids and antimicrobial proteins as well as cytokines and chemokine to eradicate infection in concert with other defense components of the body. PRR: Pattern recognition receptor for microbial products; AML: Antimicrobial lipids; AMP: Antimicrobial proteins. Other sources: Other defense cells recruited to the site of infections such as macrophages and neutrophils.

wide range of lipid classes is separation by thin layer chromatography with colorimetric visualization with a variety of reagents. Reversed phase high performance liquid chromatography with evaporative light scattering detection allows for more quantitative studies. Definitive and highly quantitative analysis is achieved with mass spectral analysis usually combined with gas chromatography or liquid chromatography. There are several web sites (accurate at the time of printing) that offer extensive hands-on information regarding lipid handling and analysis. These include the Cyberlipid Center (http://www.cyberlipid.org/), The American Oil Chemists' Society Lipid Library (http://lipidlibrary.aocs.org/), and the Lipidomics Gateway (http://www.lipidmaps.org/). Furthermore, some lipid manufacturers offer a wealth of technical support. Those who would like to take on the challenge of lipidomics will fare well by identifying a collaborator with a background in biochemistry and expertise in mass spectrometry and metabolomics.

While lipid extraction protocols are well established with one of the most frequently used one dating back to Bligh and Dyer[93], a major hurdle in investigating functional properties of AMLs, in particular nonpolar lipids like cholesteryl esters, is their low solubility in aqueous media used for antimicrobial activity testing. For FFA addition of low concentration of ethanol such as 0.05% allows for solubilization. However, for less polar and non-polar lipids embedding of the lipid of interest in liposomes prepared from various phospholipids has been proven successful for *in vitro* studies[6,94,95].

CONCLUSION

AMLs as effectors of the innate immune response and microbial counter strategies are an emerging field of study. New investigators are invited to enter the field to uncover the regulation of AML production, their delivery to pathogens and mechanism of action. We hope that this review has piqued the interest and will usher new investigators to this challenging and growing field.

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EDITORIAL

Epigenomic revolution in autoimmune diseases

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Abstract

Autoimmunity is believed to develop when genetically predisposed individuals undergo epigenetic modifications in response to environmental factors. Recent advances in the understanding of epigenetic mechanisms suggest, in autoimmune diseases, a multi-step process involving environmental factors (*e.g.*, drugs, stress) and endogenous factors (*e.g.*, cytokines, gender), both leading to the deregulation of the epigenetic machinery (DNA methylation, histone modifications, miRNA), that in turn specifically affects the immune system and/or the target organ(s). Such effect is reinforced in those patients with risk variants mapping to epigenetically-controlled regulators of immune cells. As a consequence, autoreactive lymphocytes and autoantibodies are produced leading to the development of the autoimmune disease. Potential new therapeutic strategies and biomarkers are also addressed.

Key words: Autoimmunity; Epigenetics; Epigenetic drugs; DNA methylation; Immunology

Core tip: The present editorial focuses on recent progress made in elucidating the relationship between environmental factors, epigenetics, genetics and the pathogenesis of autoimmune diseases (AID). Because of their primary function, epigenetic mechanisms offer potential advantages in terms of prevention, diagnosis, and treatment of complicated diseases such as AID.

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INTRODUCTION

Autoimmune diseases (AID), which jointly affect 5% to 10% of the general population in developed countries, are the third leading cause of morbidity. Onset of AID occurs from childhood to late adulthood and disproportionately affects women at all ages. AID can affect persons of all racial, ethnic, and socioeconomic groups, although the impact of racial background varies among AID. Over 80 different AID have been characterized and they are defined by the presence of autoreactive lymphocytes (T and B) and autoantibodies. Since the 1950s, AID have been divided into two classes: organ-specific AID in which the immune-mediated injuries are localized to a single organ or tissue, such as the pancreas in type 1 diabetes (T1D); and non-organ-specific (systemic) AID in which the immune reactions are directed against many different organs and tissues resulting in widespread injury, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and primary Sjögren's syndrome (pSS)[1]. No definitive diagnostic markers exist for most AID and classification criteria (but not diagnostic criteria) were introduced to circumvent this difficulty in order to categorize the patients into groups. However, classification criteria suffer from many limitations, one of which is a lack of sensitivity since, in some cases, it takes one to two decades to verify the status relative to the criteria. In fact, AID should be viewed as an "immunological disease continuum" driven first by a deregulation of the immune system and leading, in the end, to a classic AID[2]. Another important limitation of this classification is related to the therapeutic responses, which can be extremely variable from one patient to another, especially when highly specific new biotherapy treatments are used, thus suggesting that a specific AID can result from several pathways.

At the beginning of 2015, the ENCODE program (Encyclopedia of DNA elements) has shown the close connection between epigenetic factors, genetic factors and the different immune cell subsets[3]. Indeed, Farh *et al*[3] demonstrated that susceptibility genes associated with AID were concentrated, not at the level of protein-coding genes, but rather in non-coding regions (90%). These new regions were characterized and it was revealed that they were the major target of epigenetic modifications sensitive to endogenous factors, and were, in particular, specific to one or more immune cell subsets. As a consequence (Figure 1), the development of AID can be viewed as a multi-step process that involves environmental and endogenous factors, both leading to deregulation of the epigenetic machinery, which in turn specifically affects the immune system and/or the target organs. The cellular impact of the epigenetic dysregulation can be amplified in the case of genetic mutations. As a consequence autoreactive lymphocytes and autoantibodies are produced leading subsequently to the development of the AID.

ENVIRONMENTAL FACTORS

Several lines of evidence strongly support a critical and pathogenic role for environmental factors in AID development. An elegant way in which to evaluate the environmental factor hypothesis consists of determining the disease concordance rate (CR) between monozygotic twins (MZ). Applied to several AID, the use of MZ twins has been decisive in characterizing those AID with high environmental components, such as systemic sclerosis (SSc; CR 5%) and RA (CR 5%-20%), compared to AID with high genetic components, such as Crohn's disease (CD; CR 75%-85%) and psoriasis (CR 40%-65%). In between, SLE (CR 24%-57%), pSS (CR 15%-25%), T1D (CR 30%-50%) have an intermediate CR, indicating a scenario in which genetics and environmental factors are equally involved[4].

MZ twin studies discordant for AID have further demonstrated the importance of epigenetic modifications in the affected twin. Applied to SLE, studies have revealed that peripheral blood leukocyte CpG methylation profiles are demethylated in MZ twins discordant for SLE[5]. Differences in DNA methylation between unaffected and affected twins were also reported using peripheral lymphocytes from SSc and primary biliary cirrhosis patients[6,7], in T cells from psoriasis patients[8], and in monocytes from T1D patients[9]. Such observations have to be interpreted in relation to the observations that DNA methylation patterns and histone acetylation profiles diverge with age in healthy MZ, with the greatest differences in those who have distinctly different lifestyles[10]. Direct evidence has been provided previously that drugs, UV light, cigarette smoking as well as chemicals can induce important epigenetic changes[11].

ENDOGENOUS FACTORS

Targeting the proinflammatory cytokines of the innate immune system, such as TNF-alpha and IL-6, has been very effective in management of AID. T cell and B cell directed therapies, such as the anti-CD20 monoclonal antibody (mAb) B-cell-depleting agent rituximab, are also effective[12,13]. Treating patients with biotherapy has powerful influence on the epigenetic machinery. In RA, treating patients with anti-TNF-alpha mAb therapy was shown to increase the histone acetyltransferase/histone deacetylase (HAT/HDAC) ratio through the control of HDAC1, whereas rituximab increased nuclear activity of both HAT and HDAC[14]. Similarly, we have observed that treating pSS patients with rituximab restores global DNA methylation in salivary gland epithelial cells[15], and that using anti-IL6 receptor mAb (itolizumab) repairs the defective DNMT1 pathway and DNA methylation in SLE B cells[16].

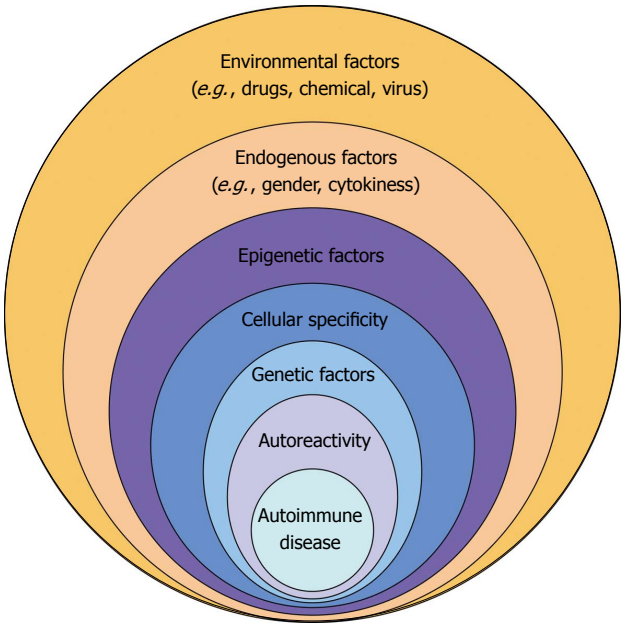

Figure 1 Multiple factors are necessary to promote autoimmune diseases in genetically predisposed individuals.

EPIGENETICS

The pioneering argument supporting epigenetic dysregulation in AID was provided by analyzing the effects of two drugs, procainamide, used to treat cardiac arrhythmias, and hydralazine, used to treat hypertension, which are associated with a 5% and 20% incidence risk for developing drug-induced lupus, respectively. It has been demonstrated for both drugs that they interfere with CpG DNA methylation directly (procainamide) by inhibiting DNMT1, the main DNA methyl transferase, or indirectly (hydralazine) by blocking the MEK/Erk activation pathway that controls DNMT1 expression[17]. Another argument to consider with regards to epigenetic dysregulation is related to the abnormal detection of retrotransposons in AID[18,19]. Retrotransposons, which comprise 45% of the genome, are known to affect the human genome in different ways: first by generating insertions or modifications into protein-coding regions that can result in genetic diseases; second by introducing long-range gene regulatory elements (enhancer, repressor/silencer, insulator); third by producing transposon-derived proteins with antigenic and/or immunosuppressive functions; and, in some cases, by promoting fusion proteins[20,21]. One example is related to the human T cell leukaemia related endogenous retrovirus (HRES-1) that is inserted in the long arm of chromosome 1 at position 1q42. HRES-1 expression is controlled at the epigenetic level by DNA methylation[22] and an association between SLE and HRES-1 polymorphisms has been described[23]. When expressed, HRES-1 has the capacity to produce a p38gag protein that can, in turn, induce the development of Abs as observed in 52% of patients with SLE in contrast to 3.6% in healthy donors[24].

CELLULAR SPECIFICITY

Defects in CpG DNA methylation are usually seen in AID with the particularity that distinct cell-types are involved. In pSS, DNA demethylation involves epithelial cells with a reduction of DNMT1 and an increase of Gadd45alpha[15]. In SLE, T cells, B cells and monocytes are demethylated due to a decrease in the enzymatic activity of DNMT1 and DNMT3a, and an increase in the enzymatic activity of Gadd45alpha and MBD4[25]. In RA, synoviocytes are demethylated which is associated with a reduction in DNMT1, MBD2 and the methyl donor S-adenosylmethionine (SAM). In contrast, DNA hypermethylation has been described in endothelial cells from SSc patients and keratinocytes from patients with psoriasis. Changes in histone modifications are also detected in AID. Global H3 and H4 hypoacetylation and hyper H3k9 trimethylation, plus a negative correlation of H3 acetylation with the disease activity, characterize $CD4^+$ T cells from SLE patients. In synovial cells from RA patients, HDAC1 overexpression has been demonstrated.

GENETICS

The first revelation of strong associations between AID and genetic factors was established with the human leukocyte antigen (HLA) region. The HLA super-locus is present in chromosomal position 6p21, spanning approximately 4 megabases, and contains 132 protein-coding genes composed partly of the classic HLA class I (HLA-A, -B, and -C) and class II (HLA-DP, -DQ, and -DR) genes which are involved in antigen processing and presentation to CD8 and CD4 T cells, respectively. Furthermore, the HLA locus is characterized by an exceptionally high degree of polymorphism, and most of the HLA variants characterized to date have relative associations with specific AID when present.

Moreover, with the development of the genome-wide association study project, up to one hundred non-HLA genetic associations were characterized in AID[26]. The list is not exhaustive as next-generation sequencing technologies contributed to the characterization of rare single nucleotide polymorphisms (SNPs) and additional SNPs, copy number variations and microsatellites associated with AID. Non-HLA gene associations are not disease specific and odds ratios (OR) are usually modest (1.1 to 1.8) in contrast to the HLA genes (OR, usually > 2). Another important point is the distribution of the AID-associated causal genetic factors that are present within protein coding regions (5%), splice junctions (0.2%), promoters (8%), 3' untranslated region (UTR, 3%) and, last but not least, within cell-specific long range gene-regulatory sequences (60%)[3].

AUTOREACTIVITY

Lymphocyte differentiation starts in the bone marrow from hematopoietic multipotent stem cells, and specific epigenetic signatures accompany each stage.

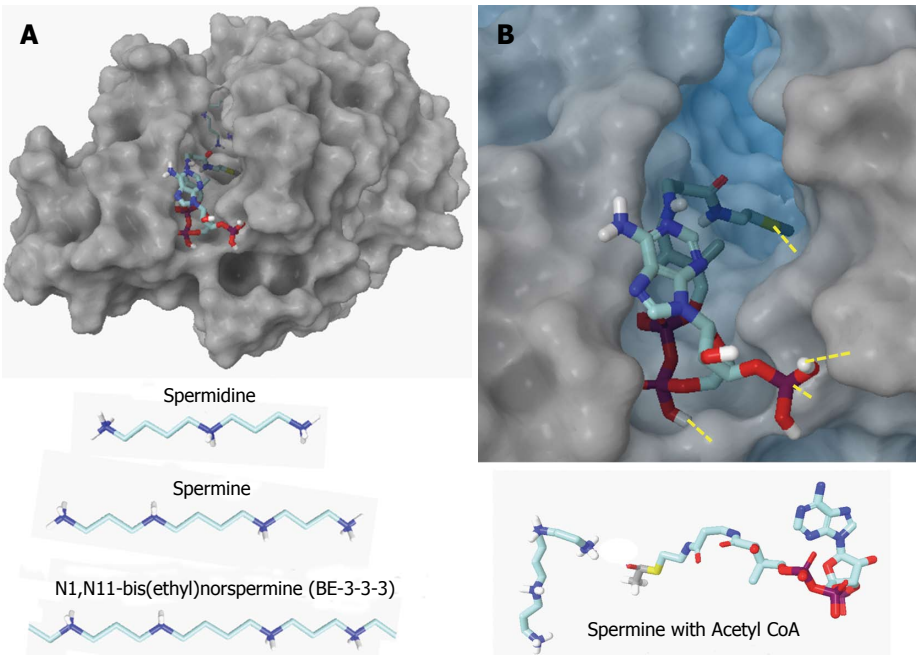

Figure 2 Human wild type spermidine/spermine N1-acetyltransferase. Spermidine/spermine N1-acetyltransferase (SAT1) functions as a dimer with two channels in which the acetylation reaction occurs. A: Top – SAT1 with spermine and acetyl CoA docked in one channel. Bottom – the normal substrates, spermidine and spermine, are shown along with a known inhibitor, BE-3-3-3; B: Top - Close-up of acetyl CoA docked in the channel. Yellow dashes denote hydrogen bonds. The substrate will enter the other end of the channel and bind. Bottom – Spermine and acetyl CoA bind in close proximity in SAT1 so that the acetyl group can transfer to the substrate at which point CoA is released and the acetylspermine has reduced affinity so it releases. Structure of SAT1[38] is 2B4D.pdb from the Protein Data Bank (www.rcsb.org).

Thus, coordinated transcriptional regulation by DNA methylation/demethylation and histone post-translational modifications (*e.g.*, H3K4me3, H3K27me3) is necessary for cytokine polarization in T helper cells, CD8 cytotoxic T cell differentiation, the selection of regulatory T cells and possibly B cell autoreactivity *via* rearrangement of the antigen receptor gene[27,28]. Indeed, blocking DNA methylation with specific inhibitors in activated B- and T-cells leads to the emergence of autoreactive lymphocytes and development of a SLE/pSS-like disease when passively transferred to normal mice[29,30]. The implication of DNA methylation in the control of lymphocyte autoreactivity is reinforced by the analysis of lymphocytes in the immunodeficiency centrometric region instability and facial anomalies syndrome, which is characterized by a non-functional DNMT3b[31]. In these patients, there is an absence of mature T cells and accumulation of immature B cells with an autoreactive B cell receptor.

THERAPEUTIC POSSIBILITIES

The beneficial effect of HDAC inhibitors (HDACi) has been demonstrated in several autoimmune models. When used in the spontaneous lupus mouse model MRL/lpr/lpr, trichostatin A, a known inhibitor of HDACs, leads to a decrease in renal disease by modulating cytokines, but the autoAb production was not affected[32]. HDACi ameliorates other autoimmune animal models, including: the rat collagen-induced arthritis; the multiple sclerosis model, experimental autoimmune encephalomyelitis; and the T1D model. In addition, it is suspected that epigenetically modified chromatin complexes represent an important immunogenic stimulus leading to autoantibody production[33].

Another recently identified therapeutic set of epigenetic targets in AID is spermidine/spermine N1-acetyltransferase 1 (SAT1) involved in the polyamine pathway which can entail excessive consumption of the cell's methyl donor, SAM, by the S-adenosylmethionine decarboxylase (AMD1)[34,35]. Both SAT1 and AMD1 are elevated in synovial fibroblasts from RA patients while, at the same time, DNA methyltransferase (DNMT1) and global DNA methylation are decreased[36]. Blocking SAT1 activity with specific siRNAs restores DNA methylation[37]. Therefore, development of specific SAT1 inhibitors is emerging as a new therapeutic approach. Computational tools can greatly accelerate drug discovery targeting SAT1 since crystal structures of wild type human SAT1 are available along with known inhibitors as references, such as N1, N11-bis(ethyl)norspermine (BE-3-3-3) with fairly well understood mechanisms of inhibition (Figure 2).

To date, among the five epigenetic drugs approved by the food and drug administration, only one, tofacitinib, a Jak1/2 inhibitor that controls histone phosphorylation, has been proven efficacious in RA. Several Jak inhibitors are currently being tested in AID, and more than 100 epigenetic drugs are in various stages of development. However, the main limitations of these drugs in chronic diseases such as AID are related to the relative lack of specificity, the lack of cellular specificity, the limited activity, as well as the risk of major side effects. As

a consequence, new epigenetic medications need to circumvent these limitations. Furthermore, the development of epigenetic biomarkers may also help screen high-risk populations before the onset of the disease for prevention, and to provide a rationale to select responder patients that can benefit from epigenetic drugs.

CONCLUSION

The arguments presented here indicate that epigenetic changes precede AID and confer risk for AID suggesting a strong argument for epigenetic causality in genetically predisposed individuals. As a consequence, better understanding of the pathways leading to epigenetic deregulation would undoubtedly have benefits for prevention, follow-up and treatment of AID.

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EDITORIAL

Stem and immune cells in colorectal primary tumour: Number and function of subsets may diagnose metastasis

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Abstract

An important percentage of colorectal cancer (CRC) patients will develop metastasis, mainly in the liver, even after a successful curative resection. This leads to a very high mortality rate if metastasis is not detected early on. Disseminated cancer cells develop from metastatic stem cells (MetSCs). Recent knowledge has accumulated about these cells particularly in CRC, so they may now be tracked from the removed primary tumour. This approach could be especially important in prognosis of metastasis because it is becoming clear that metastasis does not particularly rely on testable driver mutations. Among the many traits supporting an epigenetic amplification of cell survival and self-renewal mechanisms of MetSCs, the role of many immune cell populations present in tumour tissues is becoming clear. The amount of tumour-infiltrating lymphocytes (T, B and natural killer cells), dendritic cells and some regulatory populations have already shown prognostic value or to be correlated with disease-free survival time, mainly in immunohistochemistry studies of unique cell populations. Parallel analyses of these immune cell populations together with MetSCs in the primary tumour of patients, with later follow-up data of the patients, will define the usefulness of specific combinations of both immune and MetSCs cell populations. It is expected that these combinations, together to different biomarkers in the form of an immune score, may predict future tumour recurrences, metastases and/or mortality in CRC. It will also support the future design of improved immunotherapeutic approaches against metastasis.

Key words: Colorectal cancer; Metastasis; Stem cells; Immune surveillance; Dendritic cells; Prognosis; Flow cytometry; Lymphocytes; Regulatory cells

Core tip: Metastasis relies on differentiation of some cancer stem cells in the primary tumour niche led by many micro-environmental signals. These signals include the participation of immune cell subsets such as tumour-infiltrating lymphocytes, dendritic cells and regulatory populations. Metastatic stem cells can be identified in the removed primary tumour. The study of the number and function of these immune

cell populations in parallel with metastatic stem cells (MetSCs) in the primary tumour, together with follow-up data of patients, will define the usefulness of specific immune and MetSCs cell population combinations. This can be combined with defining new biomarkers as future predictors of tumour recurrences, metastases and/or mortality in colorectal cancer.

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INTRODUCTION

Even when a primary tumour has been perfectly removed by surgery, at the moment of diagnosis tumour cells may have disseminated and established themselves in distant locations (metastasis). Metastasis accounts for the vast majority of deaths from cancer. Metastasis is a complex phenomenon developing through several stages, such as the intravasation of cancer cells from the primary tumour, dissemination through the circulation and extravasation in different organs, survival on arrival and settlement into latency, and reactivation, division and colonization of the organ, generating a new macroscopic tumour.

Migrant cancer cells that manage to settle in a distant tissue are known as disseminated tumour cells (DTCs)[1]. However not all DTCs are able to generate a new macroscopic tumour and those having such potential are called metastatic stem cells (MetSCs)[2]. The properties that support the survival, self-renewal, dormancy, and reactivation of these MetSCs have been recently reviewed[2], with the most remarkable conclusion being that MetSCs cells have been identified as are cancer stem cells (CSCs).

Most cancers display a hierarchical organization that resembles that of their tissue of origin. CSCs is the only cell type there with long-term self-renewal potential, the microenvironment niche sustaining this potential. They are the phenotypic and functional equivalent of normal stem cells but with the inconvenience of having acquired oncogenic mutations. Both CSCs and non-CSCs can display a migratory behaviour at the invasive front of primary tumours frequently associated with an epithelial to mesenchimal transition. MetSCs may derive from non-stem cell DTCs that reacquire the competence to initiate tumour growth after a period of latency, however this process of phenotypic plasticity is neither totally accepted nor well-understood[2-4]. However, the majority of extravasation and settlement survivors in the host tissue that endow tumour-initiating capacity (*i.e.*, MetSCs) are CSCs[5].

It can be deduced from the data above that MetSCs-cells harbouring the signaling pathways capable to initiate metastasis- already exist in the primary tumour and MetSCs can be tracked from the removed primary tumour. This approach would be specifically important for metastasis diagnosis since it is becoming clear that metastasis does not particularly rely on driver mutations. Therefore, genomic biomarkers are not actually useful for metastasis diagnosis. Environmental and tumour environmental signals do provide the epigenetic amplification for cell survival and self-renewal mechanisms[6].

METASTATIC STEM CELLS IN COLORECTAL CANCER

Colorectal cancer is the third most prevalent tumour worldwide. In developed countries, around a 30%-50% of patients who were through a successful curative resection still relapse or develop metastases, mainly in the liver. These patients show a very high mortality rate if those metastases are not detected early[7].

In CRC many lines of evidence support that MetSCs are already present in the primary tumour. A first line of evidence comes from marking of tumour cell populations with lentivirus, which has allowed the clonal analysis of human colorectal cancer (CRC) cells, showing that metastases arise from primary tumour cells that display long-term self-renewal capacity, are quiescent, and resistant to chemotherapy (*i.e.*, CSCs)[8,9].

A second line of evidence comes from experiments with genetic mouse models. Upon acquiring activating mutations in the Wnt pathway, intestinal stem cells generated adenomas[10]. Another lineage-tracing analysis showed that a stem cell population resembling those present in normal intestinal mucosa not only sustained the long-term growth of these benign lesions[11-13], but also of late stage CRCs and even liver metastases[14-16]. In mice, cell populations characterized by the expression of stem cell markers isolated from human primary tumour samples (CRC and other epithelial tumours) were capable of generating metastasis when transplanted[17-19]. The last line of evidence comes from the clinic, since high expression of adult stem cell markers in primary tumours have been associated with poor prognosis and metastatic relapse[14,15,18,19].

BIOMARKERS OF METASTATIC STEM CELLS

An important current question is which stem cell markers should be used for CSCs characterization and whether MetSCs are in fact a CSCs subset that can be tracked using present knowledge.

We have just demonstrated that soluble CD26 levels (sCD26) are a much better serum biomarker for the detection of CRC metastasis or tumour recurrence when compared to other markers in clinical use such as CEA, CA-19.9 or CA-72.4[7] levels. At the same time, others have demonstrated the relationship

between the presence of $CD26^+$ cells, detected by immunohistochemistry in primary CRC tumour biopsies and prognosis of metastasis[20]. It is plausible these results are related to the $CD26^+$ CSC population capable of generating metastasis when transplanted in mice[18]. This population comprised $CD133^+$, $CD44^+$ and $CD26^+$ cells isolated from the primary tumour. However, although they majorly encompass the known features of CSCs, they were not the only CSCs present in the primary tumour biopsies. In fact, due to plasticity in CD133 and CD44 expression, these markers do not seem the most appropriate at least as MetSCs markers[21-24]. Another candidates for CRC MetSCs characterization have been described including CD166, CD29, CD24, Lgr5, EpCAM, ALDH1, CDCP1, CXCR4, CC188[21,23] and ephrin type B receptor 2 (EphB2)[25], although many of these markers are also expressed in normal colonic stem cells (*i.e.*, Lgr5, ALDH1, or CD29) complicating the distinction between CSCs and normal stem cells. Despite this, most of these markers are co-expressed in the primary tumour, so it is expected that a particular biomarker combination can be used to identify MetSCs in CRCs. This will help to understand the function of these CSCs and identify new therapeutic targets as well as to play a significant role in clinical disease management[26]. From our present knowledge, CRC MetSCs should be found among the high-expressing Wnt targets $Lgr5^{++}$ and $EphB2^{++}$ cell population[25,27] also co-expressing $CD133^+$ and $CXCR4^+$, markers of a well known metastasic cell population with a recently discovered autofluorescent subcellular compartment[4]. This autofluorescence results from the accumulation of riboflavin in ATP-dependent ABCG2 transporter-coated vesicles exclusively located within the cytoplasm of cells across different human tumour types with CSC features[4]. It is possible that CD26, intriguingly related to some extent to the CXCR4/SDF-1 axis[28], could also be included among these markers.

METASTATIC TRAITS IN PRIMARY TUMOURS

As mentioned above, cell subsets with gene expression signatures to mediate dissemination, survival capability on arrival to distant organs, and entering a dormant state in many cases[2,6-8] before metastatic spreading, have been repeatedly identified in primary tumours[4,25-28]. These traits may be used to predict future relapses before dissemination.

However, (1) there is only a very small percentage of cancer cells with these properties; and (2) these cells are originated by the epigenetic amplification caused by many supporting pathways[2,6]. Little is known about these pathways despite its major clinical importance, since killing latent MetSCs by depriving them of that support seems the most attractive therapeutic approach.

A likely site for selection of metastatic traits in primary tumours is at the invasive front, the intersection of an advancing tumour mass and the surrounding stroma. Cancer cells at the invasive front of primary tumours are exposed to the stresses of invading surrounding tissue, of hypoxia, and of the immune surveillance. This complex milieu includes cancer-associated fibroblasts (CAFs), newly generated blood vessels[29], tumor-associated macrophages, myeloid progenitor cells, and blood platelets. Various stromal cell types produce cytokines such as Wnt, Notch, tumour necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), hepatocyte growth factor and hedgehog, which support the survival and fitness of CSCs[16,30-32]. Under selective pressure, these signals skew the heterogeneous cancer cell population towards a preponderance of clones primed for survival, self-renewal, invasiveness, migration, and the stress of infiltrating distant tissues (*i.e.*, future MetSCs).

In fact, it seems that when the stroma of a primary tumor is rich in cells and signals resembling those of a particular distant tissue, cancer cell clones selected in this primary tumour could be primed to thrive in that particular tissue[2,29,30]. For example, cells and signals in a colorectal gut tumour resembling the liver environment will induce metastasis of this CRC in the liver[33].

At the same time, some already cited tumour-derived soluble factors together with other signals such as VEGF, SDF-1, IL-10, and enzymes such as indoleamine 2, 3-dioxygenase or cyclooxygenase-2, or the adenosine pathway, are well known factors responsible for the expansion of induced-T regulatory T cells (iTreg) in tumour-bearing hosts[34-37] as well as for inducing the accumulation of immature dendritic cells (iDCs), which in turn promote the expansion of iTreg[38]. Both phenotypically and functionally, iTreg cells are distinct from natural Treg (nTreg) and accumulate both in tissues and peripheral blood of cancer patients. These iTreg are presumably responsible for the suppression of anti-tumour functions of immune cells migrating to the tumour site, thus promoting tumour escape from the host immune response[35].

IMMUNE SURVEILLANCE IN COLORECTAL CANCER

From the point of view of the three immune hallmarks of cancer stating that tumours (1) are able to thrive in a chronically inflamed microenvironment; (2) can evade immunorecognition; and (3) are able to suppress immune reactivity[39], CRC is particularly known for the many evidences connecting tumourigenesis and inflammation, such as the decreased incidence of tumours in individuals under non-steroidal anti-inflammatory drug treatment, the increased incidence of tumours in overweight patients, and its relationship with commensal bacteria. We have reviewed recently these facts altogether affecting inflammation both locally and systemically[40].

According to this activation of the immune system, cells of the innate immune system such as neutrophils[41],

macrophages[42], natural killer (NK) cells[43] or DCs[44] as well as cells of the adaptive immune system such as $CD4^+$ helper and $CD8^+$ cytotoxic T lymphocytes (CTLs)[45,46] accumulate in sites of CRC development. Although immune cells release inflammatory mediators (see above) with proangiogenic and prometastasic effects[47] to the reactive stroma, at the same time tumour-infiltrating lymphocytes (TILs) in CRC have been shown to inhibit tumour growth and are associated with improved prognosis[46-52].

The concept of cancer immunoediting[53] has been divided into three phases namely elimination, equilibrium and escape[54]. In the elimination phase or cancer immunosurveillance, immune cells detect and eliminate transformed cells but this elimination could be incomplete in which case some tumour cells remain either dormant or continue to evolve accumulating further changes that can modulate the expression of tumour-associated antigens (TAAs) or other factors that increase their fitness. During this time the immune system still exerts a selective pressure eliminating some transformed clones but if this elimination is again incomplete, the process results in the selection of tumour cell variants (MetSCs among them) which are able to resist, avoid or suppress the anti-tumor response, leading to the escape phase[54].

It has been shown that CRC induces an immunosuppression state, marked by reduced secretion in patients of several cytokines such as IFN-γ or TNF-α by monocytes/macrophages. As this immunosuppression was reversible after resection of the affected tissue[55], this data held the promise of immunologically targeting tumour cells, provided the mechanisms of immune escape and tumour-induced immune suppression are overcome.

T CELLS

As previously mentioned, human CRC tissue is infiltrated by a variety of immune cells often in the margins of the transformed tissue, in the invasive front. Several studies have characterized the lymphocyte infiltration of CRC and confirmed the concept of prognostic impact of these TILs[45,56]. In most cases, the lymphocytes infiltrating the cancer tissue, and most frequently the area along the invasive margin, are either $CD4^+$ and/or $CD8^+$ T cells[57].

Despite their low numbers, $CD8^+$ T lymphocytes infiltrating the neoplastic epithelium are positively correlated with longer disease-free survival time[52,56-58] and in fact, the density of T $CD8^+$ and $CD45^+$ lymphocyte infiltration was recently shown to have a better prognostic value than the classic tumor node metastasis classification factor[59]. Previous data have shown that these TILs have antitumor activity[60,61], and some TAAs have been identified as potential targets of cytotoxic $CD8^+$ T lymphocyte responses[60-62]. Later, T cell responses against mutated normal antigens such as those of the microsatellite instable (MSI) subgroup of CRC or the small subgroup of tumours with no signs of MSI but also with high mutational load were detected[50,63,64]. Coherently, the microsatellite instability-high phenotype present in 15% of early CRC confers good prognosis[50,65]. Therefore these so-called tumour-specific somatic mutations are potentially the best targets for adoptive T cell therapy, although there are many open questions like how many somatic mutations create suitable epitopes[66]. However, at the same time these tumours have clearly adapted to this immune pressure because many CRCs express no or reduced levels of HLA-I[67-69]. Although this is a classical mechanism of transformed cells to avoid the host immune response[70,71], there are conflicting results regarding CRC expression of HLA class I antigens as associated with poor prognosis[72], probably because NK cells are important effectors in the anti-tumour response against CRC (see below).

During an immune response $CD4^+$ T lymphocytes can differentiate into two broad phenotypic subtypes: T helper 1 (Th1) or Th2[73,74]. These two different subtypes secrete different types of cytokines, and consequently activate different types of immune responses. Th1 lymphocytes secrete IFN-γ and TNF-α, which produce the activation of CTLs, NK cells, macrophages and monocytes, all of which contribute to a cellular immune response that is effective against tumour cells. However, Th2 lymphocytes secrete a different set of cytokines such as IL-4, IL-5, IL-10 or IL-13, all of which deviate the response to a humoral immune response, and this kind of immune response is less effective at eliminating cancer cells[73-75]. A shift towards a Th2 response has been shown in CRC patients, with reduced levels of Th1 cytokines and normal or elevated levels of Th2 cytokines, an imbalance that becomes more significant the further the disease progresses[76-78], with levels of the Th1 cytokines having a prognostic value in terms of patient survival[73].

The mechanism through which CRC cells can shift the T cell immune response could be due in part to the secretion of cytokines that inhibit the development of Th1 responses, such as TGF-β and IL-10, either by the CRC cells themselves or CAFs[79]. Among the roles assigned to TGF-β in cancer development[79-85], it has been cited the inhibition of T lymphocyte proliferation and differentiation preventing naïve T cells from acquiring effector functions[86] and the inhibition of the ability of TILs to kill cancer cells as well as tumour-specific $CD8^+$ cytotoxic responses[87], although recently discovered stromal factors such as tumour-derived exosomes carrying death receptor ligands directly contribute to apoptosis of activated effector $CD8^+$ T cells[88]. IL-10 immunosuppresses TILs[89] but this immunosuppressive effect is mainly indirect and mediated by DCs and Treg lymphocytes (see below)[90,91].

In addition, although the role of other T helper populations, Th17 and Th22, in the development of CRC is still unclear, it seems that decreased Th17 and Th22 responses are associated with the development of CRC[92].

B CELLS

Many of the TAAs identified in CRC so far, potential targets of cytotoxic $CD8^+$ T lymphocyte responses, has been done by the identification of auto-antibodies present in the plasma of cancer patients compared to healthy donors, and although the clinical significance of those serologically-defined antigens still have to be demonstrated, several are attractive candidates for cancer vaccines[60]. Interestingly, antibody responses against some TAAs correlate with $CD8^+$ responses in those patients[61,62], supporting the idea that the immune response taking place in CRC patients requires coordinated $CD4^+$, $CD8^+$ and B cell responses, turning Th2 anti-tumour responses a not so negative factor as previously supposed[73]. However, tumour-infiltrating $CD20^+$ B cells (TIL-B) have being poorly investigated despite their described positive prognostic value[93]. Engagement of tumour-reactive B cells may be an important condition for generating potent, long-term T cell responses against cancer[94].

DENDRITIC CELLS

Dendritic cells are key antigen-presenting cells that play a central role in the induction of immune responses including anti-tumour responses[95,96]. It has been shown that CRC patients have DCs infiltrating the tumour mass or the surrounding tissue forming clusters with T lymphocytes[97] and that this infiltration seems to correlate with a better prognosis[98,99]. In fact, activated and matured DCs induce an antigen-specific response leading to T cell proliferation and differentiation into helper and effector lymphocytes[100].

However, CRC tumour cells are able to impair the function of these cells. *In vivo* tumour-infiltrating DCs show an immature phenotype[101] and iDCs presenting self-antigens to both $CD4^+$ and $CD8^+$ T cells induce tolerance in those lymphocytes[102,103]. In this direction, tissue culture media from CRC explants inhibits DC maturation with reduced levels of CD54, CD86, HLA-DR and CD83, and induces IL-10 secretion while inhibiting secretion of IL-12p70, factors that inhibit Th1 immune responses and probably protect the tumour from a potent immune response[104]. Moreover, as mentioned before, iDCs correlate with infiltration and the expansion of iTregs[35,103].

NK CELLS

NK cells play a major role in the immune response to CRC[59] and are a prognostic factor[105].

NK cells are typically defective in infiltrating solid tumors with only 30% of patients showing NK infiltration and with only a 9% with more than four NK cells, as it has been shown in a large cohort[106]. Tumour cells has several mechanisms to inhibit recruitment and activation of NK cells[107-109], but this fact does not have a direct effect on tumor progression *per se*[107] probably explaining why the presence of NK cells in combination with $CD4^+$ T lymphocytes in colorectal tumours had no detectable effect on the clinical course of the disease[43,106].

However, in CRC the infiltration of both NK cells and $CD8^+$ T cells was associated with prolonged patient survival in the same study, suggesting NK-$CD8^+$ cell crosstalk in the tumor microenvironment[106]. These data agree with the fact that *ex vivo* activation and expansion of both NK and CTLs followed by their intraveneous infusion in patients with stage Ⅳ colon cancer improved their quality of life[110], or the fact that one of the mechanisms of action of cetuximab, a monoclonal antibody against the epidermal growth factor receptor widely used for the treatment of metastatic CRC (mCRC), is antibody-dependent cell-mediated cytotoxicity, triggered by Fc-gamma-R on NK cells[59,111].

Of the utmost importance is the fact that NK cells play a crucial role in preventing recurrence[112] probably because they are able to target CSCs/MetSCs[113].

REGULATORY CELLS

Treg cells characterized by the expression of CD25 and the transcription factor Foxp3 are critical for the prevention of autoimmunity and the regulation of immune responses to foreign and self-antigens[114]. Adaptive iTreg, a distinct population from nTreg, accumulate in tissues and the peripheral blood of cancer patients. In many of those human cancers high densities of such Tregs in the tumor correlates with poor disease outcome[115].

However, they are associated with an improved survival rate of CRC patients[115,116], or other carcinoma with prominent inflammatory infiltrates (*i.e.*, certain types of breast cancer), despite iTreg contrasted functionality[117,118]. A hypothesis has been put forward to explain this apparent contradiction indicating that those $Foxp3^+$ Tregs infiltrating the tumour mass were already in the healthy colorectal tissue to suppress excessive inflammation and immune responses resulting from the commensal microflora[103,119].

It has been hypothesized that these cells posses a contextual plasticity controlled and driven by the tissue microenvironment[103]. The main question is which factors or signals in the microenvironment regulate Treg functions thereby preventing adverse effects of chronic inflammation or autoimmunity[120]. It seems that the cellular content of the CRC infiltrate do that by silencing the tolerogenic pathway of plasmacytoid DCs[121]. These cells, different to myeloid DCs, additionally promote tolerance and Treg differentiation and suppressor functions in the solid tumour presumably *via* the Nrp-1/semaphorin-4^a pathway (plasmacytoid DCs are one of the major sources of semaphorin-4^a), and the infiltrate would block this pathway[120]. Thus, it is important monitoring not only for the frequency but also for the functionality of iTreg in cancer.

In addition, the presence of other regulatory populations such as natural killer T (NKT) cells or Bregs can

not be excluded since the nature of the regulatory cell types that dominate in any given tumour is not totally understood[122,123]. The role played by regulatory type Ⅰ and Ⅱ NKT cells has been studied in syngeneic mice models of colorectal and renal cancer. In those models, having both type Ⅰ and Ⅱ NKT cells or neither of them, Treg depletion was sufficient to protect against tumour outgrowth, however in those mice lacking only type Ⅰ NKT cells, Treg blockade was insufficient to protect mice pointing to an important role played by type Ⅱ NKT cells in suppressing tumour growth[123].

HYPOTHESIS

An important reduction in the level of serum sCD26 in patients with non-metastasic CRC makes sCD26 a promising candidate for a future serum screening test[124]. We have previously suggested that these altered levels in CRC could be due to alterations in the number or frequency of lymphocyte populations expressing this biomarker[7,125].

We pretend to analyze by flow cytometry the expression of CD26 in the different leukocyte cell populations mentioned above that could be identified in primary CRC tissue biopsies. These analyses will be combined in parallel with the analysis of the known markers for MetSCs in cells of the same tissue[48,126]. All this information, together with the follow-up of the patients for up to 5 years, will help to define the usefulness of different cell population combinations, both immune and CSCs, and/or biomarkers. These combinations will assemble an immune score[59,101] that functions as predictor of future tumour recurrences, metastases and/or mortality in CRC. At the same time, this increased knowledge will support a better design of future immunotherapeutic approaches against metastasis.

Moreover, from a methodological point of view, the use of flow cytometry allows very potent qualitative and quantitative multiparametric analyses, contributing with new information to classical and modern[57,58,127] anatomopathological studies where no *in situ* information is lost.

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MINIREVIEWS

GRP78 expression beyond cellular stress: A biomarker for tumor manipulation

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Abstract

Physiological stress takes place in the endoplasmic reticulum (ER) of cells where activation and up-regulation of genes and proteins are primarily induced to enhance pro-survival mechanisms such as the unfolded protein response (UPR). A dominant protein in the UPR response is the heat shock GRP78 protein. Although GRP78 is primarily located in the ER, under certain conditions it is transported to the cell surface, where it acts as a receptor inducing pathways of cell signaling such as proliferation or apoptosis. In the prolonged chronic stress transportation of the GRP78 from the ER to the cell membrane is a major event where in addition to the presentation of the GRP78 as a receptor to various ligands, it also marks the cells that will proceed to apoptotic pathways. In the normal cell that under stress acquires cell surface GRP78 and in the tumor cell that already presents cell surface GRP78, cell surface GRP78 is an apoptotic flag. The internalization of GRP78 from the cell surface in normal cells by ligands such as peptides will enhance cell survival and alleviate cardiovascular ischemic diseases. The absence of cell surface GRP78 in the tumor cells portends proliferative and metastatic tumors. Pharmacological induction of cell surface GRP78 will induce the process of apoptosis and might be used as a therapeutic modality for cancer treatment.

Key words: Cell surface GRP78; Apoptosis; Endoplasmic reticulum stress; Tumor cells; Cancer; Cardiovascular ischemia; Hypoxia

Core tip: In the prolonged chronic stress transportation of the GRP78 from the endoplasmic reticulum to the cell membrane is a major event where in addition to the presentation of the GRP78 as a receptor to various ligands, it also marks the cells that will proceed to apoptotic pathways. In the normal cell that under stress acquires cell surface GRP78 and in the tumor cell that already presents cell surface GRP78, cell surface GRP78 is an apoptotic flag. This review analyzes the input of cell surface GRP78 on apoptosis in normal and tumor cells.

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INTRODUCTION

Cellular stress response covers a number of molecular changes that cells undergo in reaction to patho-physiological conditions, such as lack of nutrients and oxygen, or exposure to toxins in their micro environment[1,2].

The cellular response to stress aims to protect the cells by either a short or a long term mechanism that minimizes the damage to the cell integrity. Cellular stress responses are primarily located in the endoplasmic reticulum (ER) and are mediated through highly conserved stress proteins such as heat shock proteins, some of which are only activated by stress while others are involved both in stress responses and in normal cellular functioning[2,3].

One such mechanism is the unfolded protein response (UPR), an evolutionarily conserved mechanism in which survival or apoptotic pathways are activated[4,5]. UPR is initiated upon the accumulation of unfolded proteins in the ER. The master UPR regulator is the glucose-regulated protein GRP78, a member of the heat shock protein 70 family that functions as a chaperone for the folding, maturation and transport of polypeptides and proteins in the ER[6,7]. GRP78 is also a key member of the UPR. Its primary role is to protect cells from undergoing apoptosis under physiological stress conditions[8]. If the adaptive response fails, apoptotic cell death ensues[9].

As an adaptive response to ER stress, the UPR triggers a set of pathways that results in the activation of inositol-requiring protein1 (IRE1), PKR-like ER kinase (PERK) and setting in motion transcription factor 6 (ATF6)[10]. Activation of these pathways selectively suppresses protein synthesis while promoting the translation of other specific proteins and regulating a variety of UPR target genes expression, including glucose-regulated protein GRP78 and the major pro-apoptotic transcription factor CHOP (also called GADD153)[10].

The induction of GRP78 to enhance protein folding and assembly in the ER leads to an increase in GRP78 in the ER compartment as well as to the promotion of GRP78 re-localization to the cell surface- where it assumes a new function as a receptor for cell-surface signaling[6].

Several possibilities for how GRP78 escapes to the cell surface in tumor cells were suggested[11]. In general, GRP78 trafficking from the ER to the cell surface is not well understood. It was demonstrated that ER stress actively promotes GRP78 localization on the cell surface, however ectopic expression of GRP78 is also able to cause cell surface relocation in the absence of ER stress[11]. There are also conflicting reports of whether GRP78 is expressed on specific tumor cell lines, such as PC-3 prostate cancer cells[12]. It is reasonable that since ER membrane is a source of plasma membrane, this form of GRP78 could be cycled to the cell surface. Studies also suggested that specific cell types may utilize different proteins for transporting GRP78 to the cell surface. For example, the ER transmembrane protein, MTJ-1 is implicated as the GRP78 carrier protein in macrophages[13]. The tumor suppressor Par-4 is reported to be required for GRP78 cell surface localization in PC-3 cells[14].

The present review aims to describe the function and modulation of cell surface GRP78 for the treatment of a number of maladies.

CELL SURFACE GRP78 IN THE NORMAL CELLS

Although Shock protein GRP78 has long been studied as a molecular chaperone in the ER expressed in mammalian cells and has a critical role in cellular integrity, its translocation to the plasma membrane on different cells was recently found to have several implications[6,15].

Stress induced mechanisms such as hypoxia that increased the expression of GRP78 on the cell surface was also found to stimulate cell cycle arrest at the G0/G1 phase, resulting in massive cell apoptosis[16]. It is possible therefore that hypoxia induced membrane GRP78 is a trigger for apoptosis.

Therefore, the binding of a peptide or an anti-GRP78 antibody to hypoxia- induced membrane GRP78 might decrease the stress protein on endothelial cell membranes and reduce apoptosis.

This last affirmation was substantially corroborated by the experiments in which cell surface GRP78 binding of peptides RoY, ADoPep1, or an anti GRP78 antibody, inhibited hypoxia-induced apoptosis of endothelial cells and induced proliferation and angiogenesis[17-19].

The addition of ADoPep1 to endothelial cells under hypoxic conditions induced a dramatic decrease in membrane GRP78 after only 15 min, as measured by FACS analysis. This was most likely due to the internalization of the cell surface GRP78 receptor. The internalization of the GRP78 receptor triggered PI3K pathway increasing Akt phosphorylation and MEK pathways including ERK phosphorylation for the activation of the survival/proliferation activity. The implications of specific inhibitors to PI3K and MEK pathways confirmed the specific signaling[18].

The inhibition of apoptosis was initiated by the internalization of the GRP78 receptor and the inhibition of cytochrome c release, caspase 3 activation and decrease of p38 phosphorylation[18-22].

Cell surface GRP78 induction in cultured endothelial cells was triggered by their incubation for 24 h under hypoxic conditions[17-19]. The process of cell surface GRP78 removal from the endothelial cells by peptide binding and its internalization, lead to the inhibition of apoptosis,

activation of a survival mechanism, proliferation and the initiation of the angiogenic process[17,23].

Similar to the results obtained with cultured endothelial cells was the outcome of experiments with ischemic (ischemia is the term for the lack of oxygen) diseases.

As for hypoxic conditions, the chronic lack of oxygen in mammalian tissues is the basis for ischemic diseases. An experimental ischemic hind limb model can be obtained by ligation of the femoral artery in one of the mouse hind limbs and comparing it to the non-operated second limb which serves as a control for the ischemic disease of the legs[17-19,24].

Histological sections from the ischemic leg that featured a significant decreased in blood flow, showed a significant increase in GRP78-positive endothelial cells along with the increased number of apoptotic cells and the decrease in number of capillaries[17,18].

A single local administration of the peptide binding GRP78 to the femoral ligated ischemic mouse alleviated the ischemia and restored blood transfusion after 3 wk. Histological analysis of the peptide treated limb demonstrated reduction in GRP78 positive endothelial cells accompanied by proliferation, numerous capillaries and restored blood perfusion[17,18].

CELL SURFACE GRP78 INDUCED APOPTOSIS IN NORMAL CELLS IS IMPLICATED IN THE PATHOPHYSIOLOGY OF SEVERAL DISEASES

Normal cells under normal conditions maintain a homeostasis of GRP78 in the ER where this protein serves as a chaperone for the normal folding and final secretion of glycoprotein[25]. Stress will induce the up-regulation of GRP78, that as stated, aims to protect the cell from undergoing apoptosis[1-4,25]. However, in chronic stress conditions the up-regulation of GRP78 expression that is associated with the expression of cell surface GRP78 on the normal cell will direct the cell to apoptosis, probably in order to protect the organism from secreting abnormal proteins[17-19,26,27].

Chronic stress conditions due to reduced blood flow in atherosclerosis or diabetes patients[28,29], might induce ischemic vascular diseases (IVD) in mammals that might affect the legs, heart and brain[19,30,31]. In pathological conditions such as atherosclerotic lesions of the human aorta and in endothelial cells of the tumor vasculature, cell-surface GRP78 co-localizes with T-cadherin in human umbilical vein endothelial cells (HUVECs). Overexpression of T-cadherin in HUVECs mediated cell survival in a GRP78-dependent fashion by increasing phospho-Akt and phospho-GSK3β and decreasing caspase-3 levels[32].

It has been demonstrated that ER stress associated apoptosis is involved in the pathogenesis of heart failure following acute myocardial infarction (MI)[30].

Ischemia was demonstrated to induce myocardial apoptosis, which results in loss of cardiomyocytes, leading to the impairment of cardiac systolic and diastolic functions[33]. In our studies, cardiomyocytes cultured for 4 h under hypoxic conditions, manifested the increased expression of cell surface GRP78 accompanied by increased apoptosis[33].

Additionally, TUNEL staining indicated apoptosis in cardiomyocytes in the ischemic myocardium model in animals. We have also found increased GRP78 staining near the infarct heart of experimentally- induced MI in mice[33].

Increased GRP78 has previously been reported in heart failure[34], in diabetic cardiomyopathy[29], and in an experimental rat coronary artery occlusion model, where the GRP78 protein level increased after short cycles of ischemia[35]. Cardiomyocytes under these hypoxic conditions manifested apoptosis[35]. As for the endothelial cells, the apoptotic process could be reversed both in cardiomyocytes cultures cells or ischemic heart tissues by the peptide binding cell surface GRP78[33]. Moreover, normal cells studies of ischemia induced increased cell surface GRP78 in neurons and ischemia of the optic nerve were conducted with similar results[36].

Cell surface GRP78 survival and apoptotic pathways in the normal cells are described in Figure 1.

CONTROVERSY OF UP-REGULATED GRP78 IN THE TUMOR CELLS

In contrast to the normal cells where cell surface GRP78 were induced by stress conditions, tumor cells were already exposed to cell surface GRP78 to a variable extent[6,21,37]. This was attributed to the tumor microenvironment that is characterized as chronic stress conditions caused by the deprivation of oxygen, glucose and nutrients[38].

The tumor microenvironment induced ER stress response activates the UPR[15,38,39] which has been shown to be up regulated in primary human tumor cells of several origins, including breast[40], lung[41], liver[42], colon[43], prostate[44] and brain[45].

Whether the UPR inhibits tumor growth or protects tumor cells by facilitating their adaptation to stressful conditions within the tumor microenvironment is still under controversy[15].

Permanently up-regulated GRP78 expression was also frequently documented in tumor cell lines and primary clinical samples[8,27]. However, it is not yet clear whether the increase in GRP78 expression facilitating tumor cell survival is achieved by the blockage of pro-apoptotic or the activation of pro-survival pathways[6,7,15].

It has been claimed that due to its pro-survival property in stress response, GRP78 contributes to tumor growth and confers drug resistance onto cancer cells[6,46]. In certain tumors the increase in tumorigenicity and drug

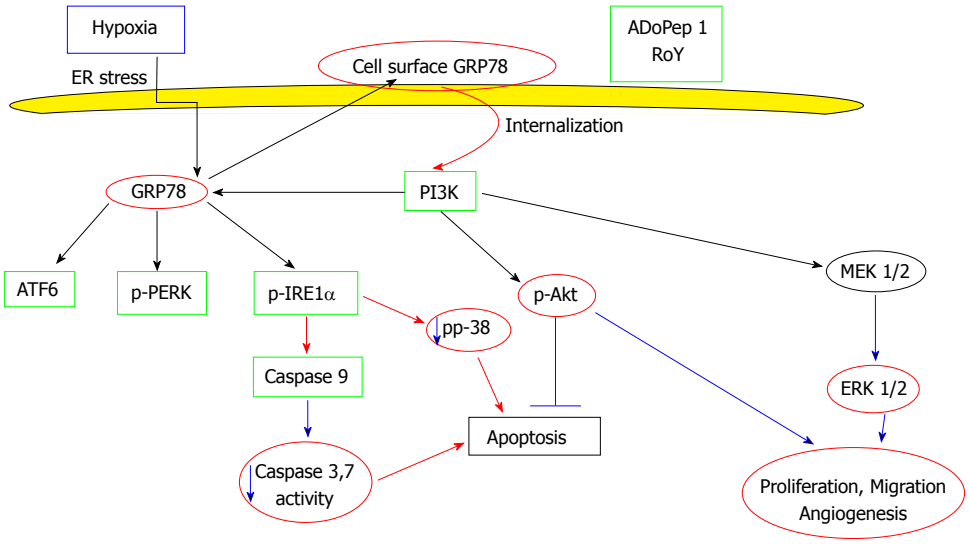

Figure 1 Cell surface GRP78 survival and apoptotic pathways in the normal cells. Hypoxia induced apoptosis is inhibited by RoY/ADoPep1 peptide binding to cell surface GRP78. Peptide binding activates the PI3K pathway by Akt phosphorylation and the NEK pathway by ERK1/2 phosphorylation, inducing endothelial cell proliferation, migration and angiogenesis. On the other hand, peptide binding to cell surface GRP78 protects cardiomyocytes and neurons from hypoxia induced apoptosis by activation of the UPR arms. This anti-apoptotic mechanism is characterized by the inhibition of p38 phosphorylation, reduced cytochrome c release followed by a decrease in caspase 3/7 activity. ER: Endoplasmic reticulum.

resistance has been attributed to the over expression of GRP78[15].

For example, it was reported that increased GRP78 expression in glioblastoma and melanoma promotes cell survival and correlates with poor prognosis[45,47]. High GRP78 levels produced the predicted result in WM266-4 melanoma and MO59J glioblastoma cell lines, reducing cell death in response to stress. However, inducing stable over-expression of GRP78 was accompanied by large changes in UPR activator expression, with reductions in PERK and increased IRE1 in glioblastoma cells but decreased ATF6 in the melanoma cells. The contribution of these changes in UPR activator expression to decreased stress sensitivity is uncertain because GRP78 over-expression in these cells was also accompanied by reduced stress, possibly as a result of the large and unexpected increases in expression of all three UPR activators[48]. In contrast, other studies associated increased GRP78 expression with tumor growth inhibition and a predictor for positive cancer treatments[49]. One such study described GRP78 as a novel positive predictor for breast cancer sensitivity to doxorubicin/taxane-based adjuvant chemotherapy[50]. Increased GRP78 expression was also shown in neuroblastoma that correlated with improved stress sensitivity and prognosis[51]. In addition, the expression of GRP78 correlated with an ameliorated prognosis in lung cancer[52].

A recent study indicated that metabolism deficiency that promotes increase in GRP78 is related to stress induced apoptosis[53]. An explanation to these contradictory reports might suggest that GRP78 has different roles as a sensor of ER stress in tumors.

Besides in the ER, GRP78 was also found to be located in the cytoplasm, mitochondria, nucleus, cell membrane as well as extracellular secretions by tumor cells[6,8].

CELL SURFACE GRP78 ON TUMOR CELLS MADIATES SIGNAL TRANSDUCTION

How GRP78 escapes to the cell surface in tumor cells is not well understood, but it may also involve some specific mechanisms adapted by the tumor cells[6].

Cell surface GRP78 was reported as a receptor to mediate tumor cell signal transduction.

Cell-surface GRP78 was found to be associates with MHC class Ⅰ, a receptor for the coxsackie A9 and Dengue viruses, and functions as the signaling receptor upon binding to the activated form of the plasma proteinase inhibitor, α2-macroglogulin (α2M*)[54]. Binding of cell-surface GRP78 with α2M* on 1-LN prostate tumor cells induced Akt phosphorylation[54] promoting cell proliferation either by inactivating apoptotic pathways or upregulating activated NFk-B. Up-regulation of NF-B augments inactivation of mitogen-activated protein kinase kinase 7 through its binding, to increase levels of growth arrest and DNA-damage-inducible β (GADD45β), thereby preventing JNK-mediated apoptosis. In addition, inactivation of apoptosis signal-regulating kinase (ASK1) by active Akt attenuates downstream JNK-mediated apoptosis[54].

Another interacting protein with GRP78 receptor is the GPI-anchored oncogene Cripto (Cripto-1, teratocarcinoma-derived growth factor 1). Cripto is expressed at high levels in human tumors and is associated with cell proliferation, migration, invasion and tumor angiogenesis *via* activation of MAPK/ERK and PI3K/Akt. Binding of GRP78 receptor to Cripto was ound to inhibit transforming growth factor-β (TGF-β) signaling and to promote cell proliferation[55].

The Protease-activated receptor 4 also known as coagulation factor Ⅱ (Par-4) is a tumor suppressor that was also associated with cell-surface GRP78. Binding of

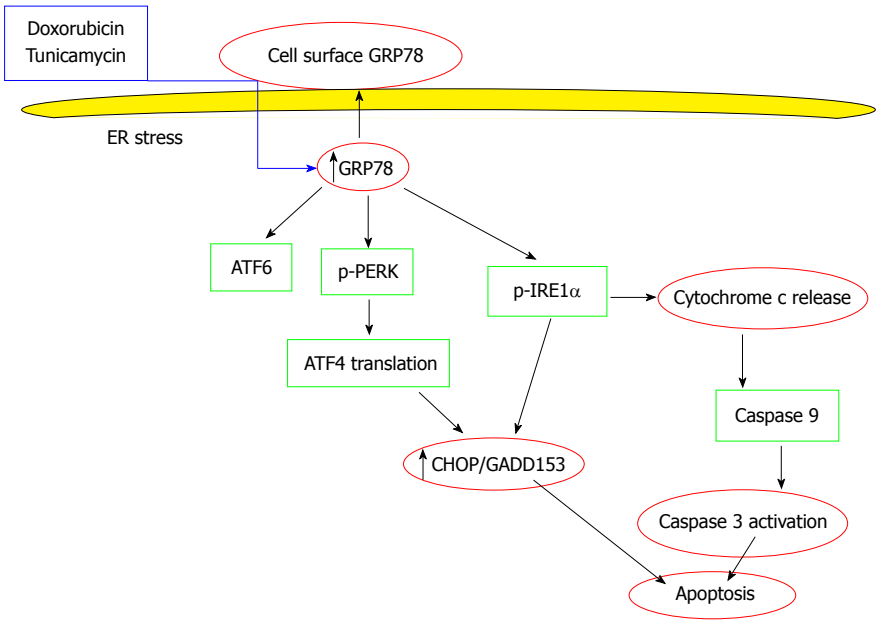

Figure 2 Cell surface GRP78 survival and apoptotic pathways in the tumor cell. Pharmacological induction of cell surface GRP78 induces apoptosis by increase expression of CHOP/GADD153, a transcriptional target of both PERK and IRE (two of the 3 UPR sensors). In parallel, doxorubicin and tunicamycin induced cytochrome c release followed by the activation of caspase 3. ER: Endoplasmic reticulum.

Par-4 to GRP78 receptor near its N-terminus elicits the apoptotic pathway by activation the FADD/caspase-8/caspase-3 pathway[14]. On the other hand, Kringle 5 of human plasminogen binding to the N-terminal domain of GRP78 receptor mediates apoptosis of tumor cells involving activation of caspase-7[56].

CELL SURFACE GRP78 ON TUMOR CELLS AS A SIGNAL FOR APOPTOSIS

Cell surface GRP78 has been demonstrated in a large variety of tumor cell types and to variable extent[11,13-15].

Subpopulations of cell surface GRP78 positive and negative were compared in order to analyze and clarify some of the contradictory conclusions on the fate of tumor cells expressing cell surface GRP78 and to elucidate whether cell surface GRP78 positive and negative tumor cells manifest different properties in colorectal cancer and whether these cells are directed to survival or to apoptotic pathways, or to pro or non metastatic directions. Two subpopulations of cell surface GRP78 positive and cell surface negative tumor cells were artificially separated by GRP78 antibody- bound magnetic beads from two different colorectal carcinoma cell lines. The HM7 cell line, a sub-line of the human colon carcinoma LS174T having a higher metastatic tendency and HCT116 cells derived from a human adenomatous polyposis. The results demonstrated that only GRP78 negative cells were highly proliferative, induced significant growth in tumor size and metastasized to the liver. In contrast, GRP78 positive cells manifested reduced proliferation, colony formation, tumor growth and liver metastases. The decreased tumorigenicity of the GRP78 positive subpopulation was abrogated by silencing GRP78 expression[57].

In breast cancer tumors, subtypes are based on the expression of cell surface receptors such as estrogen, progesterone and human epidermal growth factor receptors and tumor cells negative cell surface receptors, usually referred to as luminal and basal like tumors[58]. The luminal subtype with positive receptors has a favorable prognosis while the basal-like tumors with triple-negative receptors exhibit a poor prognosis. In addition to MDAMB468 cells which are basal- like tumor, negative for all 3 receptors were also negative to cell surface GRP78. In contrast, BT474, a representative of the luminal subtype, was also positive for cell surface GRP78 expression[59].

To evaluate the effect of cell surface GRP78 expression on the basal, receptor negative breast cancer cells, cell surface GRP78 was pharmacologically induced by doxorubicin and taxotere. These drugs significantly increased cell surface GRP78 expression on the basal receptor negative breast cancer tumor cells. Increased tumor cell surface GRP78 resulted in a significant decrease in tumorigenicity, reduced tumor growth and an increase in cell apoptosis demonstrating a direct correlation between expressed cell surface GRP78 and apoptosis. In addition, the potential application of doxorubicin and tunicamycin to induce the over-expression of cell surface GRP78 causes a significant increase in stress induced apoptosis in the triple negative tumor cell lines[59]. In a study of breast cancer, it was reported that CHOP/GADD153 over-expression correlates with a significantly lower risk of recurrence in the GRP78-positive subset[49]. It is possible that cell surface GRP78 expression is associated with the induction of the pro-apoptotic factor CHOP/GADD153.

The two major apoptotic pathways recognized as the death receptor (extrinsic) and mitochondrial (intrinsic) pathways play crucial roles in tumor progression as well as resistance to therapeutic strategies. Although the mechanisms that cause the biological selection for a specific mode of cell death remain unclear, it seems probable that the results depend on the intensity of the stress[60]. Pharmacological induction of intrinsic apoptosis was achieved by exogenous agents triggering acute ER stress[57,59].

Additional applications to cell surface GRP78 induction on tumor cells, as a potential target for cancer therapy were suggested[61]. For example, pro-apoptotic moieties or cytotoxic agents were conjugated onto peptides with a high affinity for GRP78 to successfully target and kill cancer cells[62]. Also, an un-conjugated peptidic GRP78 ligand demonstrated toxicity to prostate cancer cell by an extrinsic apoptotic pathway[63]. A human monoclonal IgM antibody against cell surface GRP78 isolated from a cancer patient was found to be capable of inducing lipid accumulation and apoptosis, probably extrinsic, in cancer cells[64].

Cell surface GRP78 survival and apoptotic pathways in the tumor cell are described in Figure 2.

CONCLUSION

The significance of cell surface GRP78 expression, beyond cellular stress, might be the focus of new therapeutic strategies for ischemic diseases. Pharmacological manipulation of cell surface GRP78 in tumor cells may serve as a new modality for tumor therapy.

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EDITORIAL

Chimeric antigen receptors: On the road to realising their full potential

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Abstract

Chimeric antigen receptors (CARs) are fusion molecules that may be genetically delivered *ex-vivo* to T-cells and other immune cell populations, thereby conferring specificity for native target antigens found on the surface of tumour and other target cell types. Antigen recognition by CARs is neither restricted by nor dependent upon human leukocyte antigen antigen expression, favouring widespread use of this technology across transplantation barriers. Signalling is delivered by a designer endodomain that provides a tailored and target-dependent activation signal to polyclonal circulating T-cells. Recent clinical data emphasise the enormous promise of this emerging immunotherapeutic strategy for B-cell malignancy, notably acute lymphoblastic leukaemia. In that context, CARs are generally targeted against the ubiquitous B-cell antigen, CD19. However, CAR T-cell immunotherapy is limited by potential for severe on-target toxicity, notably due to cytokine release syndrome. Furthermore, efficacy in the context of solid tumours remains unproven, owing in part to lack of availability of safe tumour-specific targets, inadequate CAR T-cell homing and hostility of the tumour microenvironment to immune effector deployment. Manufacture and commercial development of this strategy also impose new challenges not

encountered with more traditional drug products. Finally, there is increasing interest in the application of this technology to the treatment of non-malignant disease states, such as autoimmunity, chronic infection and in the suppression of allograft rejection. Here, we consider the background and direction of travel of this emerging and highly promising treatment for malignant and other disease types.

Key words: Adoptive T-cell immunotherapy; Chimeric antigen receptor; Genetic engineering; Leukaemia; Cancer

Core tip: Adoptive immunotherapy using chimeric antigen receptor-engineered T-cells has been in development for 25 years and, recently, has achieved striking impact in the management of B-cell malignancies. However, the therapy is often accompanied by significant toxicity, in particular cytokine release syndrome. While efficacy in B-cell acute leukaemia provides important clinical proof of concept, this therapy remains unproven in the arena of solid tumours and other disease types. Furthermore, manufacture of cell products is complex and difficult to scale out for widespread clinical use. Significant effort on all of these fronts will be required to enable this promising immunotherapy to enter the therapeutic mainstream.

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CHIMERIC ANTIGEN RECEPTORS: THE ROAD TO CREATION

During the latter half of the 20th century, several key advances in our understanding of human immunology have set the stage for the recent emergence of effective immunotherapies for cancer[1]. The pace of this advance is illustrated by the fact that, prior to 1960, the immunological role of the thymus was completely unknown. Thereafter, a cascade of research led to the discovery of two distinct lymphoid cell types of Thymic and Bone marrow origin - namely T- and B-cells - and established their unique roles in the respective establishment of cellular and humoral adaptive immunity. T- and B-lymphocytes proved to have an extraordinarily diverse capacity for antigen recognition, owing to recombination events that generated distinct clonotypic receptors in individual cells. In the case of the B-cell receptor, it emerged that native antigen was engaged directly and often with high affinity. By contrast, a very different system of antigen recognition was uncovered that applies to the predominant circulating T-cell population. These cells were found to interact using a T-cell receptor (TCR) with antigen-derived processed peptide fragments, presented within the groove of a human leukocyte antigen (HLA) on the target or antigen presenting cell surface. Investigation of the HLA gene system demonstrated that it is encoded within a super-locus in which classical transplantation genes are distributed into two major regions, known as HLA class Ⅰ and class Ⅱ. These genes proved to be the most polymorphic ever identified - hindering the clinical development of allogeneic transplantation and cell therapy - and were shown to restrict antigen presentation to two mutually exclusive T-cell subsets that express CD8 or CD4 co-receptors respectively. The discovery of cytokines such as interleukin-2 (IL-2) subsequently proved pivotal in enabling the *ex-vivo* culture and *in-vivo* support of such T-cells for adoptive immunotherapy[2]. As molecular immunology evolved, it became apparent that the ability of antigen to elicit T-cell activation was a complex and titratable phenomenon in which signalling was integrated *via* a multi-molecular synapse containing the TCR/CD3 complex (signal 1) and one or more co-stimulatory receptors, such as CD28 or 4-1BB (signal 2)[3]. More recently, the existence of a counterbalancing family of co-inhibitory receptors has been established, best exemplified by CTLA-4 and programmed death (PD)-1[4].

In parallel with these basic advances, a deepened appreciation has emerged of the dynamic inter-relationship between human cancer and the host immune system. In the 1980s, it was shown that tumour-infiltrating lymphocytes could elicit cytolytic activity against autologous melanoma[5]. Proof of concept for therapeutic activity of *ex-vivo* expanded TIL cells in patients with melanoma followed shortly thereafter[6], in a manner that was potentiated by preparatory lymphodepletion[7]. The increasing appreciation of how transformed cells are subject to such immune surveillance has recently been acknowledged in the proposal that evasion of this process represents a fundamental hallmark of cancer[8]. Two ground-breaking translational outputs of this growth in understanding have emerged more recently, rendering cancer immunotherapy the "Breakthrough of the Year" in 2013. The first of these involves immune checkpoint blockade - a form of immunotherapy that aims to release anti-tumour T-cells from the suppressive effects of co-inhibitory receptor ligation. This rapidly developing therapeutic approach has achieved striking and durable responses in patients with an ever-increasing number of solid tumour types[9]. The second approach aims to target precisely the cytotoxic ability of T-cells through the introduction of an ectopic TCR or chimeric antigen receptor (CAR). Use of TCR engineered T-cells has achieved clinical efficacy in small numbers of patients. However, the approach is limited by the fact that T-cells remain HLA restricted, rendering universal utility problematic and compromising activity against transformed cells that have downregulated HLA antigen

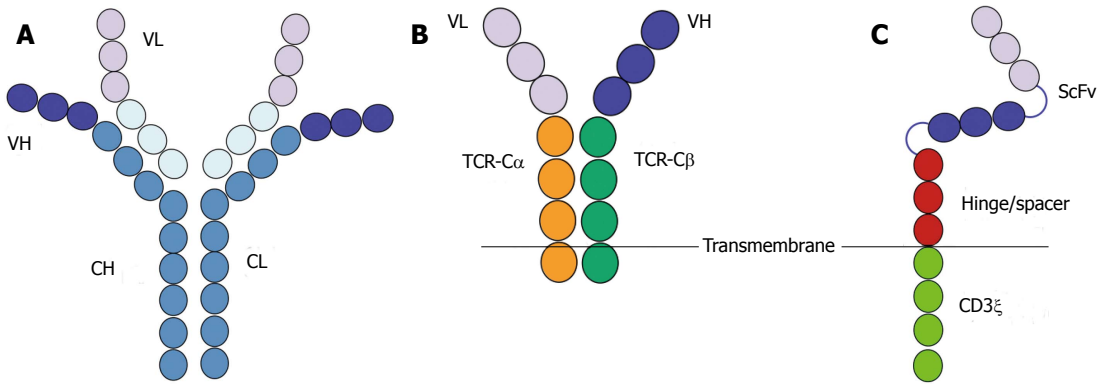

Figure 1 Chimeric antigen receptor structure. A: Most commonly CARs are targeted using the VH and VL domains of a monoclonal antibody; B: In prototypic CAR designs, these were individually fused to the constant domains of TCR-Cα and TCR-Cβ; C: However, more recent iterations entail the initial fusion of VH and VL, thereby creating a scFv moiety which is then fused to a hinge/spacer and a T-cell activating module, such as the endodomain of the ζ subunit of the TCR/CD3 complex. CH: Constant heavy domains; CL: Constant light domains; CARs: Chimeric antigen receptors; VH: Variable heavy; VL: Variable light; scFv: Single chain variable fragment.

expression. Use of CAR-engineered T-cells addresses these obstacles although additional issues continue to hinder this approach, most notably limited efficacy against solid tumours and toxicity due to cytokine release syndrome (CRS). The focus of this editorial is to assess prospects for CAR-based immunotherapy in this emerging new era of cellular therapy.

EVOLUTION OF CAR MODELS THROUGH THE GENERATIONS

CARs are synthetically engineered, membrane-spanning fusion proteins that can engage target molecules in their native form. First described by Eshhar in 1989[10], CARs comprise a targeting moiety, which is coupled to a signalling domain *via* hinge/spacer and transmembrane elements. This was originally achieved by individually combining the variable region of the heavy (VH) or light (VL) chains of a monoclonal antibody (Figure 1A) to the constant regions of the α or β subunits of the TCR (Figure 1B). Since this required the co-expression of two subunits, the initial structure was later simplified such that a single-chain variable fragment comprising a fusion of the VH and VL domains were joined to γ or ζ chains that respectively provide signalling from Fc receptors or the TCR/CD3 complex (Figure 1C)[11]. Alternatively, ligands may be used to engage single or multiple target species, broadening specificity[12]. Expression of CARs may be achieved using integrating viral vectors, notably retrovirus or lentivirus, or through transient non-viral systems (*e.g.*, mRNA electroporation). Identifying the most appropriate host cell population (or combination) for CAR T-cell immunotherapy, such as $CD8^+$, $CD4^+$, naïve, central memory, memory stem T-cell or natural killer cell is an area of intense investigation at present. Using a sequence of *ex-vivo* cell culture techniques, individualised autologous cell-based therapies can be engineered for use in patients (Figure 2).

CARs have evolved through a series of iterative modifications, designed to enhance potency and clinical benefit (Figure 3). First generation CARs possess only one intracellular domain and had limited T-cell activation capacity, leading to little clinical activity in early trials. Mindful of the key role of co-stimulation in the optimal activation and survival of T-cells, fusions were next produced in which modules from CD28 or 4-1BB were placed upstream of CD3ζ. Such second generation CARs mediate enhanced proliferation, cytokine release, *in-vivo* persistence and therapeutic efficacy in comparison to their predecessors[13-15]. Next, third generation CARs were engineered that contain two co-stimulatory modules[16] and which are now being evaluated in clinical trials, although the jury is still out with regard to the magnitude of this advance. Much effort has also been directed at optimising the ectodomain, hinge/spacer region and transmembrane element used in CAR construction as well as the vectors and methods used for T-cell transduction (Figure 2)[1,17]. In parallel, CARs have been co-expressed with accessory molecules to enhance safety (*e.g.*, drug-inducible suicide genes, such as inducible caspase 9[18]) or to improve further their anti-tumour activity. Examples of the latter include the use of cytokine receptors and their derivatives that enable *ex-vivo* expansion of CAR T-cells and which confer responsiveness to tumour-associated cytokines, such as IL-4 or colony-stimulating factor-1[19-21]. Alternatively, CAR T-cells may be engineered to home to the tumour microenvironment and to acquire resistance to immuno-suppressive mechanisms that operate there, for example through constitutive or inducible production of IL-12[22] or engineered resistance to transforming growth factor-β (*e.g.* NCT00889954; https://clinicaltrials.gov/ accessed 22-6-2015).

CLINICAL EVALUATION: WHAT'S ALL THE FUSS ABOUT?

Proof of concept for the game-changing potential of CAR-based immunotherapy has been exemplified by several recent clinical trials in patients with B-cell

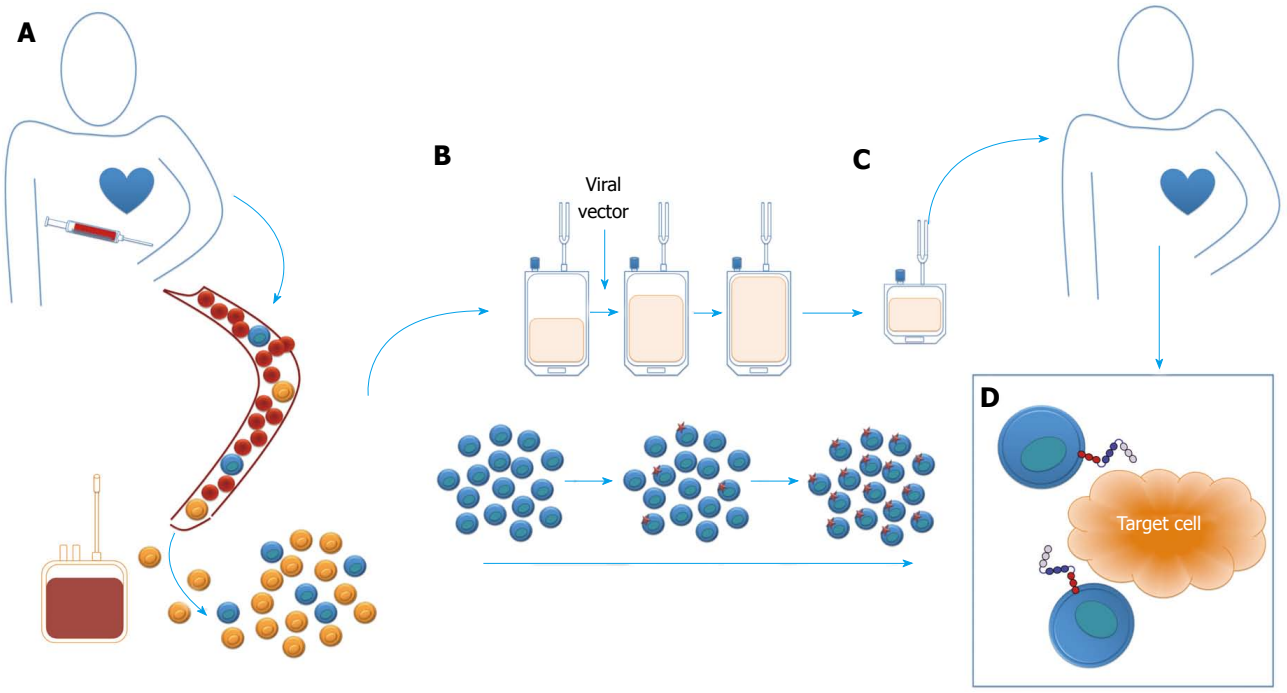

Figure 2 The overview of manufacture of chimeric antigen receptor T-cell products. A: Starting material is most commonly a leukapheresis, although some systems employ whole blood at this stage; B: Peripheral blood mononuclear cells are isolated and T-cells then activated and genetically engineered (in this case with a viral vector); C: After *ex-vivo* expansion, the cell product is formulated and administered; D: After which engineered cells can engage target cells *via* the CAR. CAR: Chimeric antigen receptor.

malignancies. In all cases, patients have received autologous CAR T-cells targeted against the ubiquitous B-cell antigen, CD19, and administered intravenously after lymphodepleting immunosuppression. In patients with acute lymphoblastic leukaemia (ALL), complete remission rates of 80% or above have been reported in several independent United States centres, employing second generation CARs that contain either CD28 or 4-1BB motifs in addition to CD3[23-27]. It is important to emphasise the unprecedented magnitude of these responses in the context of first in man evaluation in patients with otherwise untreatable malignancy. Furthermore, some of these patients had failed treatment with potent antibody derivatives such as blinatumomab, highlighting the greater potency of the CAR-based approach[25]. Highly impressive clinical outcomes have also been observed using CD19-targeted CAR T-cells in the setting of chronic lymphocytic leukaemia, and non-Hodgkin's lymphoma[23,28].

THE CLINICAL JOURNEY THUS FAR - ACHIEVING BALANCE AND CONTROL

The first comprehensive report of clinical testing of CAR T-cell immunotherapy is less than 10 years old[29]. Over that period, we have witnessed both the enormous clinical promise of this technology, together with its potential to cause extreme toxicity (summarized in Table 1). Many patients who have responded to CD19-targeted CAR T-cell immunotherapy have experienced CRS, accompanied in some cases by failure of one or more vital organs and/or macrophage activation syndrome. Pre-clinical studies indicate that such macrophage activation and release of monokines such as IL-6 represents a double-edged event, since this is required for maximal clinical efficacy[30], but contributes substantially to clinical manifestations of CRS[31]. As might be expected, anti-cytokine therapies such as the anti-IL-6 receptor antibody, tocilizumab and the anti-tumour necrosis factor-α antibody, infliximab have been used in patient management, as have traditional agents such as corticosteroids[32]. The inducible caspase 9 suicide system may also find particular utility in this setting since it has been shown to eliminate 90% of engineered cells within 30 min after administration of the triggering dimeriser drug[33].

By contrast to B-cell malignancy, efficacy of CAR T-cell immunotherapy in patients with solid tumours has been much more modest. Nonetheless, some responses have been achieved in paediatric patients, without unacceptable toxicity[34]. Solid tumours impose several key challenges to the successful development of CAR based immunotherapy that do not apply in B-cell malignancy. The first of these is the paucity of "CD19-like" targets - in other words, targets that are exclusively expressed on tumour cells alone, or in addition to tissue(s) that are dispensable or which perform a function that can be bypassed using a pharmacological solution. Consequently, self-antigens that are over-expressed in tumour cells and found at low levels in healthy tissues are generally selected for CAR T-cell

Table 1 Summary of toxicities that may be attributed to immunotherapy using chimeric antigen receptor engineered T-cells

On target on tumour toxicity	
Cytokine release syndrome[32]	Exaggerated activation of multiple leukocyte subtypes Marked elevation in circulating levels of multiple cytokines Pyrexia and acute phase response Vascular leakage Failure of one or more major organs May be related to tumour burden Occurs days to (exceptionally) weeks after T-cell infusion
Macrophage activation syndrome[25]	Haemophagocytosis Organomegaly Elevation of ferritin, aminotransferases, lactate dehydrogenase and triglycerides Hypofibrinogenemia
Tumour lysis syndrome	Rapid tumour cell destruction leading to profound metabolic disturbances, including hyperphosphatemia, hyperuricaemia, hyperkalaemia, hypocalcaemia and/or renal failure
On target off tumour toxicity	
CAR T-cell mediated immune attack of healthy tissue that express cognate target	Exemplified by B-cell aplasia induced by CD19-targeted CAR T-cells[23-28] hepatotoxicity induced by carbonic anhydrase IX-targeted CAR T-cells[37] pulmonary toxicity induced by HER2-targeted CAR T-cells[38]
Antibody mediated toxicity	Exemplified by anaphylaxis induced by mesothelin-targeted CAR T-cells[42]
Off target off tumour toxicity	
Insertional mutagenesis	Not seen with gene-modified T-cells as yet, unlike haemopoietic stem cells
Replication competent virus	Not seen with modern vector systems

CAR: Chimeric antigen receptor.

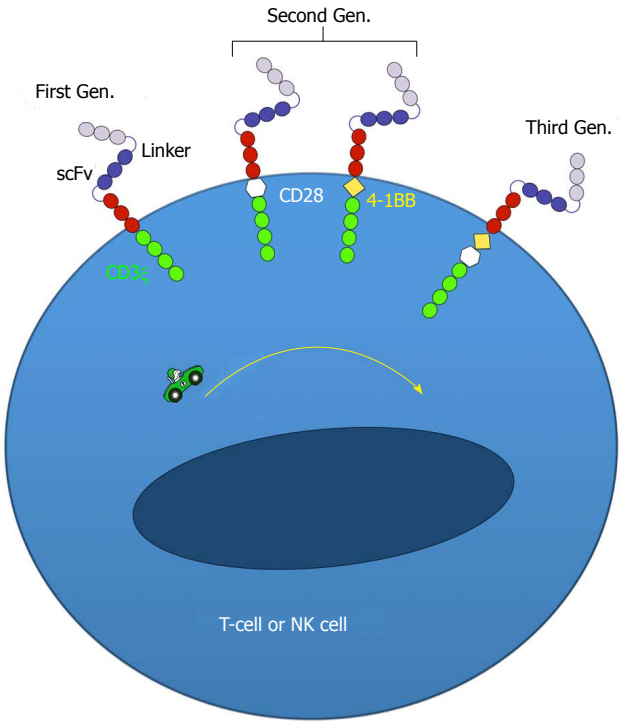

Figure 3 Evolution of chimeric antigen receptors. First Gen. CARs contain a source of "signal 1" alone, exemplified by CD3ζ. This is augmented in a second generation CAR by the inclusion of one co-stimulatory module, commonly from either CD28 or 4-1BB. The inclusion of two such co-stimulatory modules defines third generation CARs. CARs: Chimeric antigen receptors; Gen.:Generation.

immunotherapy. Indeed, targets that are expressed on T-cells, such as CD5, have been successfully targeted in pre-clinical models of T-cell lymphoma[35]. The use of lower affinity targeting moieties may enhance the safety of this approach, enabling better discrimination by CARs between tumour cells (target high) and healthy tissues (target low)[36]. Nonetheless, previous clinical experience has highlighted the difficulty inherent in negotiating this fine line[37]. One particularly unfortunate fatal adverse reaction occurred in a patient treated intravenously with 10 billion T-cells engineered to express a third generation HER2-targeted CAR, administered following lymphodepletion[38]. On-target toxicity ensued rapidly following engagement of HER2 in the pulmonary microvasculature, perhaps owing to the propensity of infused CAR T-cells to impact for several hours at that site[39]. More recently, a more cautiously implemented trial targeting HER2 has been completed and has achieved an efficacy signal without significant toxicity. Moreover, a study targeting mesothelin has also recently been reported in abstract form and, once again, has demonstrated safety, but with limited efficacy[40]. A further key challenge is the immunogenicity of CARs when administered to (non B-cell depleted) recipients[41], owing to the presence of xenogenic or other foreign sequences. The resultant antibody response favours the rapid clearance of these cells and has even resulted in life-threatening anaphylaxis[42]. An additional important obstacle is the need to enable T-cells to home more efficiently into tumour deposits and, once there, to operate effectively within the hostile tumour microenvironment. Pre-clinical approaches designed to address these obstacles are alluded to briefly above. One clinical strategy which may address some of these issues is direct delivery of CAR T-cells to the site of disease, a strategy that has recently been evaluated in glioblastoma[43] and is undergoing evaluation in head and neck cancer[20]. A summary of clinical trials in

Table 2 Clinical trials of chimeric antigen receptor T-cell immunotherapy of haematological malignancy

Target	No. of trials	Diseases
CD19	40	Acute and chronic B-cell malignancies Hodgkin's lymphoma
CD30	4	Hodgkin's lymphoma; CD30+ non-Hodgkin's lymphoma
CD20	2	B-cell malignancy
CD22	1	B-cell malignancy
Kappa light chain	1	B-cell malignancy Multiple myeloma
CD33	1	Acute myeloid leukaemia
CD138	1	Multiple myeloma
CD123	1	Acute myeloid leukaemia
Lewis Y antigen		Acute myeloid leukaemia Myelodysplastic syndrome; Multiple myeloma
NKG2D stress ligands	1	Acute myeloid leukaemia; Myelodysplastic syndrome; Multiple myeloma
Combinatorial	1	CD16-containing "universal CAR" targeted with rituximab for B-cell malignancy

CAR: Chimeric antigen receptor.

haematological malignancy (Table 2) and solid tumours/other diseases (Table 3) that are currently registered on the clinicaltrials.gov website is provided. Trials were based in United States (65), China (15), United Kingdom (5) and 1 each in Japan, the Netherlands, Australia, Sweden, Singapore, Switzerland and Israel.

THE NEED FOR AUTO-MANUFACTURING OF CAR T-CELLS

Manufacture, formulation and certification of autologous CAR-based cell therapy products continues to pose obstacles to robust large-scale production and widespread clinical use[44]. Products must be made to Good Manufacturing Practice standards, meaning that manufacturing protocols are often time-consuming, expensive, labour-intensive and cumbersome. Autologous cell products constitute the ultimate "personalised medicine" since each batch generally provides one or more treatments for a single patient. As a result, there is a need to "scale-out" to maximise the numbers of batches produced, rather than "scale-up" in order to generate large volume batches for multi-patient use. Manufacturing techniques in current use often introduce inter-operator variability, entail the use of open processing systems and require intensive training of personnel. The facilities, equipment, staffing and documentation involved in the engineering and delivery of a single product renders widespread implementation challenging, even in the setting of resource-rich countries. Regulations used to guide production and release were originally developed for traditional pharmaceutical agents with linear supply chains and well established business and supply models. These impose significant challenges for autologous CAR T-cell therapies, which involve circular supply chains in which the first step entails a blood draw or leukapheresis. Adding to complexity, there is a lack of harmonisation in the application of regulations pertaining to manufacture across different geographical sectors. In Europe for example, products require qualified person certification prior to release whereas this requirement does not operate in the United States.

In light of these considerations, efforts need to focus upon standardisation and automation of production, employing existing infrastructure such as exists in blood banks or stem cell facilities[45] and increased reliance on closed manufacturing processes wherever possible[46]. A key dilemma in this regard is the decision to opt for centralised *vs* multi-site manufacture. While the latter can minimise distance between the patient and site of production, it requires the establishment and maintenance of comparability of cell product quality across sites. This in turn emphasises the need for careful characterisation of cellular source material and raw materials. Product stability is also a key factor, which may require development of validated cryopreservation techniques, raising issues about whether products should be thawed immediately prior to shipping or subject to manipulation upon receipt at the site of administration.

An alternative manufacturing solution that is attracting increasing interest entails the development of allogeneic and potentially universally applicable CAR T-cell products. In light of HLA polymorphism and the cross-reactive nature of the TCR (leading to alloreactivity), this requires consideration of strategies to eliminate risk of graft *vs* host disease and rejection of infused cells. Although at an early stage, interest has been raised in the use of genomic editing tools to address such limitations[46]. Alternatively, use of lymphoid precursors that complete thymic education in the recipient, or engineered T-cells derived from induced pluripotent stem cells may warrant consideration as potential solutions to the histocompatibility problem[47].

DRIVING CARS INTO DIVERSE APPLICATIONS

As experience with CAR T-cell immunotherapy of malig-

Table 3 Clinical trials of chimeric antigen receptor T-cell immunotherapy of solid tumours and other diseases

Target	No. of trials	Diseases
HER2	7	Glioblastoma
		HER2 expressing solid tumours[1]
GD2	7	Neuroblastoma[34]
		GD2-expressing malignancy
		Osteosarcoma
		Melanoma
Mesothelin	4	Pancreatic cancer[40]
		Ovarian cancer
		Mesothelioma[2]
CEA	4	Breast cancer
		CEA expressing malignancy[1]
Folate receptor-α	1	Epithelial ovarian cancer[29]
EGFr	2	$EGFr^+$ malignancy
		Glioblastoma
EGFr variant Ⅲ	2	Glioblastoma
Carbonic anhydrase Ⅸ	1	Renal cell carcinoma[37]
Prostate-specific membrane antigen	2	Castrate-resistant prostate cancer
Interleukin-13 receptor α2	1	Glioma[43]
CD171 (L1 cell adhesion molecule)	1	Neuroblastoma
Extended ErbB family	1	Head and neck cancer3[20]
Fibroblast-activation protein	1	Mesothelioma
Glypican-3	1	Hepatocellular carcinoma
Pathogenic T-cell receptors	1	Type 1 diabetes[4]

(https://clinicaltrials.gov/accessed August 26th, 2015 search terms: T cell gene cancer; chimeric and cancer; T-cell cancer and gene). [1]One Trial terminated due to toxicity; [2]Trial includes the co-administration of CD19-targeted CAR T-cells to minimize anti-CAR antibody production; [3]Intra-tumoural route in use to minimize toxicity; [4]CAR targeted using a peptide + HLA-CD3ξ fusion; CAR: Chimeric antigen receptor; CEA: Carcinoembryonic antigen; EGFr: Epidermal growth factor receptor.

nant disease has grown, increasing consideration has been given to the development of CAR-based therapies for diverse non-malignant disease states. Several preclinical studies have demonstrated the potential benefit of the adoptive transfer of purified regulatory T-cells (Tregs) in the treatment of autoimmune disease and donor graft rejection. Currently, many clinical trials are investigating the safety and efficacy of adoptive transfer of *ex-vivo* expanded Tregs in these disease settings, although cells are not targeted in many cases, which may compromise efficacy. It has therefore been suggested that CAR-targeted Tregs may have a potential role achieving more potent and targeted therapeutic immunosuppression[48].

The potential benefit of T-cell based therapies in the treatment of infectious diseases, for example human immunodeficiency virus (HIV), has also been the focus of recent research and discussions. This infection is known to elicit a cytotoxic $CD8^+$ T-cell response, the potency of which is believed to correlate with viral load and disease control. This phenomenon is exemplified by a unique cohort of patients known as "HIV controllers", individuals who appear to have an inherent ability to keep the virus at bay. This phenotype is believed to be due in part to a potent $CD8^+$ HIV-specific T-cell response as opposed to an inherent resistance to initial viral infection. Based on these findings the delivery of T-cell therapies including HIV-targeted engineered CAR-based therapies was postulated to be of potential benefit in the fight against HIV and in the quest to develop a cure. Studies of first generation CAR T-cells in patients with HIV infection have demonstrated the remarkable ability of these cells to persist and to home to sites of disease, calling into question the need to evaluate more modern CAR T-cell strategies in this setting[49].

THE FUTURE OF CARS: ENSURING A SMOOTH RIDE

Based upon 25 years of pre-clinical and clinical experience with CAR-based therapies, we are potentially entering into an era of rapid advancement in design and more widespread use of CAR-based therapies within a broad range of clinical settings. However, there is still much work to be done to ensure efficient and effective progress. Major challenges remain in the cancer setting surrounding issues such as target selection, maintenance of *in-vivo* survival of CAR T-cells, and the achievement of sustained but not excessive function. Future questions need to address the combinatorial use of these cells in conjunction with conventional (*e.g.*, chemotherapy, radiotherapy, tumour-targeted monoclonal antibodies) and emerging therapies, such as immune checkpoint blockade. Development of standardised and robust manufacturing solutions also presents a new challenge to commercial development. Nonetheless, the stunning and unparalleled activity of this technology in B-cell malignancy coupled with its amenability to precise and highly refined engineering emphasises the fact that top

gear for CAR T-cell immunotherapy remains tantalisingly around the corner.

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EDITORIAL

Impact of cell death manipulation on the efficacy of photodynamic therapy-generated cancer vaccines

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Abstract

The main task of cancer vaccines is to deliver tumor-specific antigens to antigen-presenting cells for immune recognition that can lead to potent and durable immune response against treated tumor. Using photodynamic therapy (PDT)-generated vaccines as an example of autologous whole-cell cancer vaccines, the importance is discussed of the expression of death-associated molecules on cancer vaccine cells. This aspect appears critical for the optimal capture of vaccine cells by host's sentinel phagocytes in order that the tumor antigenic material is processed and presented for immune recognition and elimination of targeted malignancy. It is shown that changing death pattern of vaccine cells by agents modulating apoptosis, autophagy or necrosis can significantly alter the therapeutic impact of PDT-generated vaccines. Improved therapeutic effect was observed with inhibitors of necrosis/necroptosis using IM-54, necrostatin-1 or necrostatin-7, as well as with lethal autophagy inducer STF62247. In contrast, reduced vaccine potency was found in case of treating vaccine cells with apoptosis inhibitors or lethal autophagy inhibitor spautin-1. Therefore, PDT-generated cancer vaccine cells undergoing apoptosis or lethal autophagy are much more likely to produce therapeutic benefit than vaccine cells that are necrotic. These findings warrant further detailed examination of the strategy using cell death modulating agents for the enhancement of the efficacy of cancer vaccines.

Key words: Antitumor immune response; Photodynamic therapy-generated vaccines; Cell death; Endoplasmic reticulum stress response; Reticular unfolded proteins response; Damage-associated molecular patterns; Immunogenic cell death

Core tip: It is discussed how the mode of cell death can be important to the efficacy of cancer vaccine, using the example of photodynamic therapy-generated whole-cell vaccines. With the example of several agents modulating apoptosis, necrosis or autophagy, it is argued that the strategy of using such agents for

the enhancement of the efficacy of cancer vaccines deserves serious consideration.

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VACCINE WITH TUMOR CELLS UNDERGOING IMMUNOGENIC CELL DEATH

One of the attractive adaptations of photodynamic therapy (PDT), a cancer treatment modality producing cytotoxic lesions by localized generation of reactive oxygen species produced by light-activated drugs (photosensitizers)[1], are PDT-generated autologous cancer vaccines[2]. They are made by exposing surgically removed tumor tissue (or derived tumor cells) to PDT *ex vivo* and used to vaccinate the same patient against the original malignancy[2,3].

Cell death pathways induced by PDT are dominated by apoptosis, reflecting the activity of produced reactive oxygen species, sensitivity to photodamage of anti-apoptotic protein Bcl-2, engagement of pro-apoptotic mediators such as ceramide and stress kinases, and induction of cytochrome c release from mitochondria[4,5]. Autophagy was also found to be induced in PDT-treated cells and was traced to photodamage induction in endoplasmic reticulum (ER)[4]. The effectiveness of PDT-generated vaccines in orchestrating a strong immune antitumor reaction is considered to result from the nature of PDT-inflicted oxidative stress-based insult expressed, at least in part, in the ER of treated cancer cells[6,7]. Sensors of luminal accumulation of unfolded proteins in ER (appearing as a result of PDT-mediated photooxidative damage) engender unfolded protein response (UPR) as an attempt initially to re-establish homeostasis and this is accompanied with a general arrest of protein translation (Figure 1)[8]. In situation when ER-stress remains unresolved, several signal transduction pathways become activated securing the transition from adaptive to lethal phase of ER stress response. Such induction of reticular UPR promotes the immunogenic cells death (ICD) accompanied with the expression of an abundance of various damage-associated molecular patterns (DAMPs) driving this immune reaction[8-10]. Another suggested contributing element are PDT-induced photooxidative changes in tumor antigenic repertoire that may include the emergence of highly immunogenic tumor neoantigens that could be instrumental in overcoming immune ignorance of the tumor[2].

For optimal efficacy of PDT-generated vaccines, following treatment by PDT *in vitro* tumor tissue/cells have to be left in culture for 16-24 h before used as a vaccine[3,11]. This post-PDT incubation time interval is considered necessary for the expression of cell death-associated changes on the vaccine cells before they become fully potent vaccine material. Interference with this process, for instance by masking cell surface-exposed phosphatidylserine, inhibiting the induction of heat shock protein 70 (Hsp70), or blocking apoptosis by a caspase-3 inhibitor, will affect negatively the therapeutic impact of PDT-generated vaccines[11,12]. Thus, in order to be captured by host's sentinel phagocytes and tumor antigenic material contained in them processed and presented in an optimal way for immune recognition and elimination of targeted malignancy, the PDT-generated vaccine cells have to exhibit a particular "eat me" death profile. This revelation inspired the investigation on the prospects of a strategy to enhance the potency of PDT-generated vaccines by modulating the death process in vaccine cells. The findings uncovered thus far by this investigation are summarized in Table 1. In addition to apoptosis, the agents affecting either necrosis or lethal autophagy turned out also to be capable of changing the potency of PDT-generated vaccines.

The importance of apoptotic death of PDT vaccine cells has already been made clear earlier[11]. While physiological apoptosis is immuno-tolerogenic, the type of programmed cell death triggered by oxidative ER stress (induced by PDT and certain other cancer treatments) is associated with the engagement of danger signaling pathways arranging the trafficking of ICD-affiliated DAMPs towards cellular surface and extracellular space[10]. However, not only by blocking the apoptosis but also by impairing lethal autophagy (with agents like spautin-1 in Table 1), PDT-generated tumor cells are rendered less potent source for cancer vaccine. Moreover, the opposite effect was attained by treating these vaccine cells with an autophagy-inducing agent. This reveals that the process of autophagy, which is closely connected with the reticular UPR and is presumably activated along with PDT-induced ER stress response[8,10], also promotes ICD with a consequential boost to immuno-activating potential of PDT vaccine cells. Such action capability is consistent with the paradigm of the contributing role of autophagy in danger signaling activity in dying cancer cells and participation of key autophagy proteins in the endosomal trafficking pathway[10].

In contrast to apoptosis and autophagy, the death of PDT vaccine cells by necrosis and its death receptor-instigated programmed variant necroptosis are detrimental to their therapeutic potential. This is evident from the beneficial effects rendered by vaccine cell treatment with two different necrostatins and IM-54 that selectively blocks oxidative stress-induced necrotic cell death[13] (Table 1). All these three agents, when present during the post-PDT incubation time interval

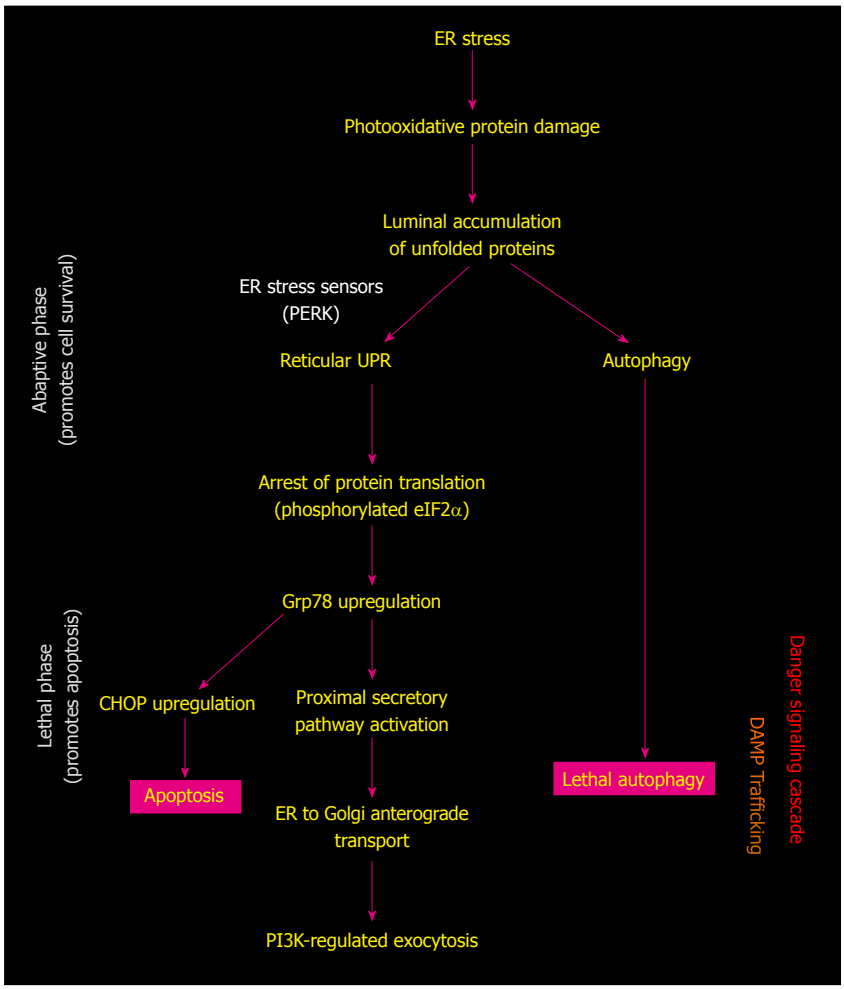

Figure 1 Molecular/biological events following photodynamic therapy-induced endoplasmic reticulum stress. PERK: Protein kinase R-like ER kinase; Grp78: Glucose-regulated protein 78; CHOP: C/EBP homologous protein; ER: Endoplasmic reticulum; UPR: Unfolded protein response.

Table 1 Changes in the therapeutic efficacy of photodynamic therapy-generated vaccines induced by modulators of cell death

Cell death modulating agent	Impact on therapeutic efficacy of PDT-generated cancer vaccine
Z-DEVD-FMK (apoptosis inhibitor)	Reduces
IM-54 (necrosis inhibitor)	Enhances
Necrostatin-1 (necroptosis inhibitor)	Enhances
Necrostatin-7 (necroptosis inhibitor)	Enhances
STF62247 (lethal autophagy inducer)	Enhances
Spautin-1 (lethal autophagy inhibitor)	Reduces

Mice with growing SCCVII tumors (murine squamous cell carcinoma) were vaccinated by SCCVII cells (20 million/mouse) that were first treated by PDT (mediated by photosensitizer ce6) and then incubated before use for 16 h with or without cell death modulating agents[3]. The therapy response was monitored by tumor size measurement in time following vaccine treatment. PDT: Photodynamic therapy.

induced an increase in the percentage of apoptotic cells in the PDT vaccine cell samples.

The above examinations on the effects of modulators of cell death by apoptosis, autophagy and necrosis/necroptosis shed novel light on the roles of cell death pathways in the generation of cancer vaccine cells by PDT. It is also becoming increasingly evident that further research is merited for elucidating the potential of agents such as autophagy promoters or necrosis inhibitors for acting as adjuvants to improve therapeutic effectiveness of PDT-generated and similar whole-cell cancer vaccines.

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REVIEW

RNA polymerases in plasma cells trav-ELL2 the beat of a different drum

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Abstract

There is a major transformation in gene expression between mature B cells (including follicular, marginal zone, and germinal center cells) and antibody secreting cells (ASCs), *i.e.*, ASCs, (including plasma blasts, splenic plasma cells, and long-lived bone marrow plasma cells). This significant change-over occurs to accommodate the massive amount of secretory-specific immunoglobulin that ASCs make and the export processes itself. It is well known that there is an up-regulation of a small number of ASC-specific transcription factors Prdm1 (B-lymphocyte-induced maturation protein 1), interferon regulatory factor 4, and Xbp1, and the reciprocal down-regulation of Pax5, Bcl6 and Bach2, which maintain the B cell program. Less well appreciated are the major alterations in transcription elongation and RNA processing occurring between B cells and ASCs. The three ELL family members ELL1, 2 and 3 have different protein sequences and potentially distinct cellular roles in transcription elongation. ELL1 is involved in DNA repair and small RNAs while ELL3 was previously described as either testis or stem-cell specific. After B cell stimulation to ASCs, ELL3 levels fall precipitously while ELL1 falls off slightly. ELL2 is induced at least 10-fold in ASCs relative to B cells. All of these changes cause the RNA Polymerase Ⅱ in ASCs to acquire different properties, leading to differences in RNA processing and histone modifications.

Key words: Interferon regulatory factor 4; Antibody secreting cells; B cell differentiation; ELL2; Secretory-specific antibody; B-lymphocyte-induced maturation protein 1; OCA-B; Super elongation complex; Xbp-1; Mammalian target of rapamycin

Core tip: B cell differentiation to antibody secreting cells is a highly regulated, complex process facilitated by factors such as interferon regulatory factor 4, Blimp-1,

OCA-B, Xbp1, and mammalian target of rapamycin. This results in a switch in immunoglobulin mRNA processing from the membrane-bound to the secretory-specific form, occurring when ELL2 releases RNAP-Ⅱ pausing during transcription elongation and causes exon skipping and proximal poly(A) site choice.

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INTRODUCTION

If a man does not keep pace with his companions, perhaps it is because he hears a different drummer. Let him step to the music which he hears….Henry David Thoreau.

B cells mature in the bone marrow, having undergone a series of DNA rearrangements to produce the uniquely rearranged immunoglobulin (Ig) molecules (H2L2) on their surfaces, the B cell receptor (BCR). Also on the B cell surfaces are: CD79 alpha and beta Ig co-activators for the BCR; CD19, which acts as a co-receptor with BCR; CD21, the complement receptor 2 for C3d; pattern recognition receptors like Toll-like receptors 2 and 4; and MHC-Class Ⅱ molecules. The mature B cells travel to the lymph nodes or the spleen and reside in niches awaiting stimulation.

Upon stimulation the B cell radically alters its program of gene expression and "hears a different drummer", turning into an antibody producing factory. If the B cells reside in and are stimulated in the marginal zone by T-independent antigen engagement of the BCRs, through the toll-like receptors, or Ig plus C3d, they will differentiate into antibody secreting cells (ASCs) with a high probability of differentiating into short-lived plasma-blasts. Activated marginal zone ASCs persist for only a few days after activation. They die rapidly either through an inability to deal with internal reactive oxygen species formed because of the large amount of secretory-specific antibody molecules they produce and/or because they fail to upregulate receptors for survival signals.

B cells that initially travel to the follicles require a more complex set of reactions in order to be stimulated by antigen. Engagement of the B cell surface CD40 occurs *via* contact with T cells carrying surface CD40 Ligand (CD154). Secretion of cytokines including interleukin (IL)-2, -4, and -5 by T cells further activates the B cells. CD40 is a member of the tumor necrosis factor superfamily of receptors and engagement results in B cell activation, isotype switching, and somatic hyper-mutation upon passing through a germinal center. Those B cells then differentiate into ASCs or memory cells. The CXCR4+ ASCs from B cells stimulated in follicles can home to specific CXCL12+ niches in the bone marrow and become long-lived ASCs. Long life for ASCs depends on soluble factors like BAFF and APRIL made by the bone marrow stroma and a touch of autophagy to repair damage in the endoplasmic reticulum (ER)[1].

TUNING UP THE BAND

How individual activated B cells choose between division, death, ASC development and class switching is unknown, and the molecular basis of this heterogeneity is still a mystery[2]. The relationship between the short-lived cycling plasma-blasts and the long-lived ASCs also remains unclear. The long-lived ASCs have the highest B-lymphocyte-induced maturation protein 1 (Blimp-1) expression, which might then explain the decreased levels of c-myc and proliferation in them[3]. We addressed these issues more fully in a recent review[4].

Regardless of the source of the B cells (MZ or FO) the activation pathways to ASCs share a number of transcription factors that alter the expression pathways and pave the way to secretion of antibody. Several genes have been implicated in both the activated B cell transcriptional network and the ASC network, such as interferon regulatory factor 4 (Irf4) and Pou2af1 (OCA-B); meanwhile, ELL2, c-Fos, Prdm1 (Blimp-1) and Xbp1 are implicated only in the ASC network[5]. Of these, only ELL2, Irf4 and Pou2af1 (OCA-B) have been shown to act directly on the *Ig* genes.

Irf4 plays a central role in B cell to ASC differentiation

Irf4, also known as Pip, is unique amongst many others within its class of Irfs. Irfs have important roles within the immune responses. Irf1 and Irf2 were the first Irfs to be recognized for their novel immunomodulation and hematopoietic effects. Studies of Irfs prompted later discovery of other members in this class. The class now includes Irf3, Irf4, Irf7 and Irf8[6,7]. Irf4 was discovered *via* analysis of the specific Ets-transcriptions factors it interacts with; Irf4 binds to PU.1, an Ets-transcription factor, and together they form a functional ternary activating complex[8].

B cells experience class-switching recombination along with particular changes to cellular Ig specific transcription factors due to Irf4 regulation. The formation and changes to the germinal center are most highly observed when centrocyte levels are decreased and Ig specific transcription factors become abundant. The progression from germinal center maintenance to germinal center-specific transcription is the final step in the B cell cascade before differentiation can occur. Irf4 meditated differentiation directs B lymphocytes to become memory B cells or ASCs[9]. ELL2, a transcription elongation factor discussed below, is highly expressed in ASCs *vs* B cells, and Irf4 binds to the ELL2 promoter to induce high levels of ELL2 mRNA[10]. When Irf4 is conditionally knocked out, germinal center formation is profoundly compromised[11]. The proximate cause of the

differentiation from B lymphocyte to ASC is Blimp-1. When Blimp-1 is upregulated, the cell is directed to differentiate. With further observation, the ultimate cause is, in fact, Irf4. Irf4 upregulation causes the downstream increase of Blimp-1 *via* PU.1, Irf4 ternary complex activation. This suggests Irf4 is the major orchestrator of B cell to ASC differentiation.

Originally, Irfs were thought to have all bound to a shared constitutive DNA consensus sequence, but later Irf4 and Irf8 were shown to have much lower affinities to these standard DNA sequence motifs. Due to the lower DNA binding affinity of Irf4 and Irf8, Ets-transcription factors are required to facilitate their DNA binding. Irf4 and Irf8 share similar Ets-transcription factor protein binding domains, and therefore the same Ets-transcription factors are used by both of them. Ets-transcription factors PU.1 and Spi-B have been shown to bind to specific DNA-binding motifs that then recruit Irf4 and Irf8[12]. PU.1 and Spi-B both promote binding to the 3' enhancers of κIg and λIg light chains. Since both Irf4 and Irf8 are recruited by these factors, there is competition between the two similar Irfs. The outcomes of the Irf competition are starkly different, since B cell to ASC transition will not occur with an abundance of Irf8[13,14].

Irf4 and Irf8 not only compete for the Ets-transcription factors, but also promote expression for repressors of the other's factors. In doing so, high levels of Irf8 would prompt decreased levels of Irf4, causing greater expression of Irf8-dependent genes. Irf8 dependent genes include *Bcl6* and *Pax5*, which are high in B lymphocytes. Irf8-dependent genes repress Aicda and Blimp-1 expression, which are products of Irf4-dependent transcription[15]. With increased Irf4, the exact opposite occurs, where Irf4-dependent genes such as Aicda and Blimp-1 are expressed. This in turn represses Irf8-dependent gene transcription. Irf8 dependent gene *Pax5* is repressed by Blimp-1, which is a negative transcription regulator in B lymphocytes. Presence of Blimp-1 represses Pax5 and c-myc. Repression of c-myc ceases cellular proliferation and causes an overall reduction of surface IgM[16]. The repression of Pax5 results in Xbp1 activation, which causes an increased production of unfolded protein response (UPR) components[17].

Irf4 has also been shown to drive Zbtb20 expression in B cells. Zbtb20, also known as Zfp288, DPZF and HOF, is a complex Bcl6 homologue that is a tramtrack, bric-à-brac, and zinc finger protein[18,19]. Ectopic expression of Zbtb20 induced terminal B cell differentiation to ACS. Along with promoting differentiation, Zbtb20 expression in plasma cells induces cell survival and blocks cell cycle progression. Zbtb20 is directly downstream and regulated by Irf4, and acts independently of Blimp-1[20].

Blimp-1 is required for ASC differentiation

Blimp-1 is encoded by the *Prdm1* gene and plays a crucial role in the differentiation of B cells to ASCs, and thus the switch from expression of membrane bound antibody molecules to secreted antibody molecules[21]. The human homolog, PRD1-BF1, was discovered by Keller and Maniatis[22] by isolating a clone from a cDNA library encoding a protein that binds to the PRD1 site of the β-IFN promoter. Its ORF presents krüppel-like zinc finger DNA-binding motifs as well as proline and acidic regions resembling those of other known transcription factors, which indicates that Blimp-1 is a transcriptional regulator[23]. Blimp-1 mRNA expression is low in B cells and only present in late stages of differentiation[23]. Through Northern blots, it has been seen that Blimp-1 accumulation increases 5-fold in cells stimulated with IL-2 and IL-5. B cells transfected with Blimp-1 mature, although not all the way, to a point of exhibiting qualities of early ASCs[23]. It was further shown that upon knocking out Blimp-1, secretion of Ig was severely reduced or failed[24]. Regardless of the levels of Blimp-1, however, B cells will not differentiate in the absence of Xbp1[25], and B cells lacking Blimp-1 are unable to normally induce Xbp1 mRNA as well as unable to normally process the Xbp1 protein[26]. This shows that Blimp-1 acts upstream of Xbp1 in the development of ASCs[25]. B cells deficient in Blimp-1, transfected with Blimp-1 on a retrovirus, were able to secrete IgM, but Blimp-1$^{-/-}$ cells transfected with Xbp1 were not, proving that Xbp1 is not able on its own to drive differentiation if Blimp-1 is absent, thus indicating that Blimp-1 plays additional roles in plasmacytic differentiation[26].

Blimp-1 blocks transcription of a large set of genes[27]. C-myc is known to block terminal B cell differentiation[28]. Ectopically expressing Blimp-1 in pre-B cell lines represses *c-myc* promoter activity[29] and causes deacetylation of histone H3 associated with the *c-myc* promoter[30]. By analyzing DNA microarrays after inducible expression of Blimp-1, it was shown that Blimp-1 represses genes associated with progression of the cell cycle as well as synthesis and repair of DNA[27]. Blimp-1 was also shown to repress the gene expression program involved in B cell identity[27]. Blimp-1 represses Pax5[27], which is known to repress Xbp1[31]. This indicates that Blimp-1 induces expression of Xbp1 by repressing its repressor, Pax5[32]. It has also been shown that Blimp-1 represses the transcription elongation factor ELL3. When the ELL3 promoter was cloned, co-transfection with *Prdm1* significantly repressed activity in B lymphocytes[33]. Blimp-1 was also seen to shut down Ig class switching by repressing expression of genes required in this process as well as inhibiting signals that serve to activate switch region Ig transcription[27].

Shaffer *et al*[27] showed that Blimp-1 participates in a negative feedback loop with BCR and Bcl6. BCR signaling restrains terminal differentiation of B cells by suppressing Blimp-1, while expression of Blimp-1 was reciprocally shown to block BCR signaling *via* the downregulation of its components[27]. Bcl6 is required for the differentiation of Germinal Center B cells[34].

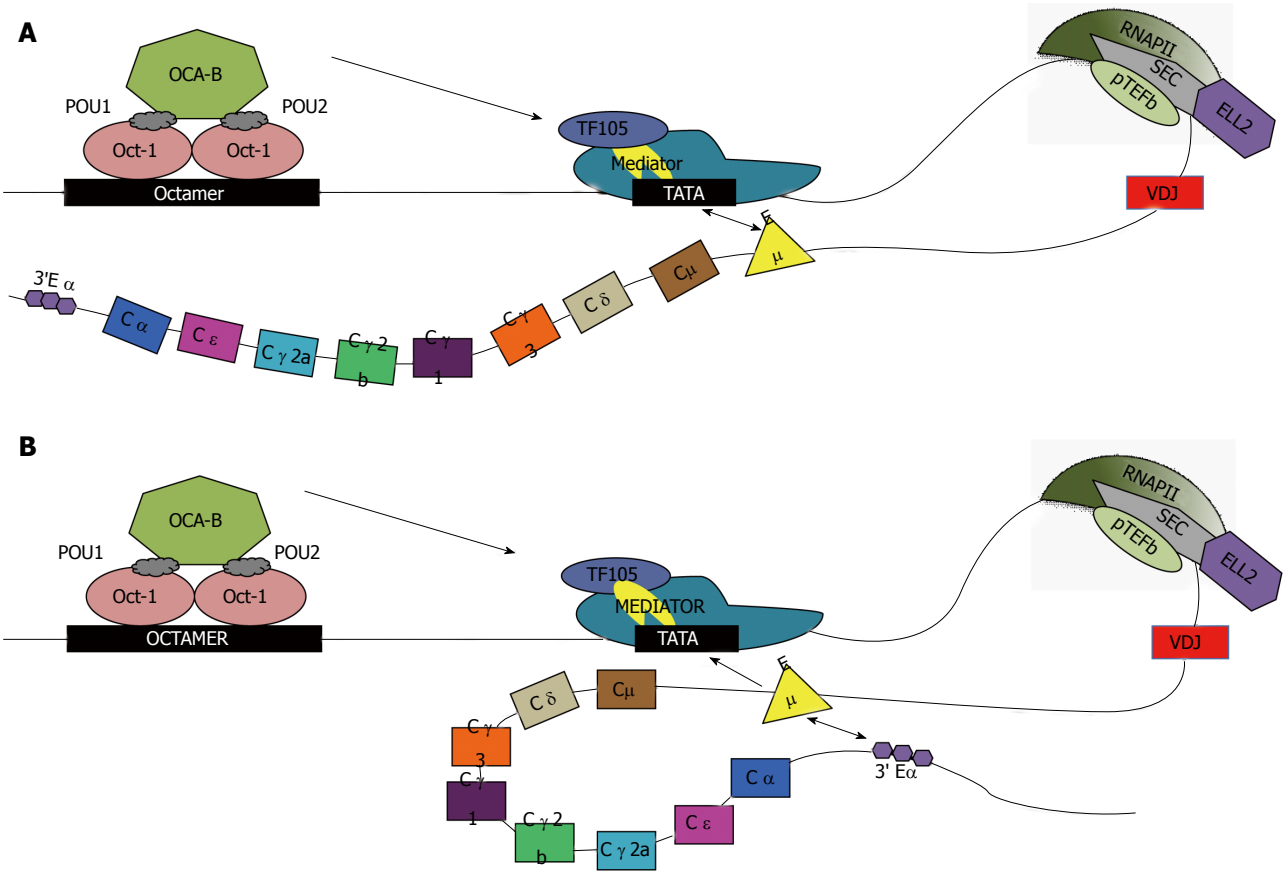

Figure 1 Transcription of Igh with alternative enhancer interactions. Oct-1 and -2 bind to the octamer sequence; OCA-B binds to Oct-2 and with TF105 (purple oval), a TFIID variant that is part of the basal transcription complex including TBP (yellow). Mediator (large teal complex) is a large complex of proteins that facilitates binding of RNAP-II to INR. Factors in Mediator like cdk8/cyclin C phosphorylate RNAP-II at ser-5 in the CTD consensus 7-mer for initiation. Then P-TEFb (cyclinT and cdk9, green oval) phosphorylates the ser-2 position of the CTD repeats of RNAP-II. Many genes have stalled polymerases awaiting the SEC, which contains ELL2 (purple hexagon) and P-TEFb[60]. Phosphorylation of the CTD by ser-5 and ser-2 is high near the Igh promoter[61]. The other members of the SEC are shown in gray. Differential transcription elongation occurs due to the potential interaction of the Igh enhancers. Cμ enhancer (annotated Eμ) interacts with promoter of Igh. A: In the first case, classical transcription of Igh can occur due to interaction between the promoter and enhancer, depicted by double arrow, without large-scale chromosomal looping; B: In the second case, along with promoter-enhancer interactions, Cμ enhancer has been hypothesized to interact with the 3' Cα enhancer (3' Eα), causing chromosomal looping. CTD: Carboxyl-terminal domain; SEC: Super elongation complex; TBP: TATA-binding protein; P-TEFb: Positive elongation factor b.

Blimp-1 also represses Bcl6, while overexpression of Bcl6 represses Blimp-1 and thus ASC differentiation[35]. This feedback loop provides very tight control over the decision of a B cell to become an ASC, for while Bcl6 is expressed in a GC B cell, expression of Blimp-1 and thus plasmacytic differentiation is blocked, but as soon as Blimp-1 is activated, Bcl6 is repressed and differentiation begins[27]. It was also shown that Irf4 deficient cells were unable to differentiate and lacked expression of Blimp-1, indicating that Irf-4 directly activates the expression of Blimp-1[36]. In addition to Irf4, c-Fos also influences Blimp-1 expression. A proto-oncogene, c-Fos is a transcriptional regulator that operates on DNA in an indirect fashion *via* its interaction with other transcriptional activators, such as c-Jun[37]. Ectopic expression of c-Fos with c-Jun induces Blimp-1 expression[38]. H2-c-Fos B cells, once stimulated with LPS, were found to proliferate at a higher level than normal B cells after LPS stimulation and induced enough Blimp-1 for terminal differentiation[39].

THE DOWNBEAT: OCA-B STARTS THE MARCH TO IG SECRETION

OCA-B aids Ig expression.

OCA-B, aka Pou2af1, BOB.1, Bob-1, OBF-1, or OBF.1, a-coactivator from B cells that increases Ig promoter transcription, was discovered by Luo *et al*[40] using the fractionation by ion-exchange chromatography of an oligonucleotide matrix-bound fraction. They isolated a novel B cell coactivator of Oct-1 and Oct-2. Binding of OCA-B to the octamer sequence of IgH is indirect and facilitated by Oct-1 and Oct-2 DNA binding[41] and as depicted in Figure 1. Oct-1 and Oct-2 contain POU domains, POU-1 and POU-2, respectively[42]. These POU domains are sufficient to mediate the interaction between Oct-1 and Oct-2 with OCA-B[41]. OCA-B has been shown to have no effect on the initial transcription of *Ig* genes, or play a role in the development of early B cells; however, mice deficient in OCA-B nonetheless exhibited impaired immune response[43].

OCA-B increases the effectiveness of Oct-1 and Oct-2 activity on Ig promoters[40]. Ectopically expressing OCA-B stimulates transcription of an IgH promoter in a HeLa nuclear extract[40]. Oct-2 mutants with deletions in one of the two activation domains were generated and were shown to have a reduction of ability to stimulate an artificial octamer-dependent promoter[41].

Several observations surrounding the phenotypes of OCA-B$^{-/-}$ mice made by Kim *et al*[44] help reveal more information involving its function. Knockouts are able to produce the same levels of IgM mRNA and protein as the WT mice, as well as produce normal numbers of mature surface IgM^{+}/IgD^{+} splenic B cells[44]. The cells had slightly reduced levels of proliferation following LPS stimulation, but the proliferative response to stimulation by anti-IgM crosslinking was greatly reduced - a response that was almost completely rescued to WT level when IL-4 was added[44]. These results suggest that B cell differentiation and expression of IgM is not affected by knocking out OCA-B, and LPS and IL-4 pathways are for the most part intact. But these mice produce reduced serum levels of secondary Ig isotypes; the numbers of surface Ig-expressing cells and IgG2b, IgG3, and IgG1 secreting cells are not different between the knockout and WT. The rates of secretion per cell, however, are much lower in the OCA-B$^{-/-}$ mice, suggesting that the knockouts are able to undergo the isotype-switching processes, but are incapable of efficiently expressing these switched *Ig* genes. Interestingly, knockout mice lack splenic germinal centers as well as germinal centers in lymph nodes[45]. There was also seen to be an increase in OCA-B expression in normal germinal center B cells[45].

Mice deficient in OCA-B also displayed a 2-4 fold decrease in splenic B cells, which suggests that it is required for splenic B cell maturation[43]. While there was a reduction in levels of mature B cells, cells of early differentiation stages remained unaffected[43]. OCA-B has been demonstrated to repress the development of the transitional Syndecan-1^{int} cell by decreasing the division-based rate of differentiation[46]. In later B cell development, OCA-B is required to promote differentiation into cells that exhibit high rates of Ig secretion[46]. This role of OCA-B in plasmacytic differentiation is in part due to its interaction with Blimp-1. OCA-B$^{-/-}$ cells do not express Blimp-1 *in vitro* in response to CD40L and IL-4, and the genes that Blimp-1 is known to repress, such as Pax5 and Bcl6, are consequently expressed at high levels in these differentiating knockout cells[46]. In addition to its interaction with Blimp-1, OCA-B has also been shown to regulate immunosuppressive miRNA expression by the conserved octamer motif in the promoter of miR-146a[47]. In the absence of OCA-B, expression of miR-146a and miR-210 is greatly reduced, an interesting finding considering both have been found to suppress NF-κB signaling[47].

A comparison of the > 100 sequences of promoter regions for Igh Ⅴ regions in the mouse genome shows only the simple consensus of an octamer binding sequence (ATGCAAT) and an INR or initiation region[48]. Some *Igh* genes contain, while some lack, a TATA box, which would be bound by TBP and basal transcription factors. OCA-B interacts with TAF105, a lymphocyte variant of TFIID[49], and is upregulated in ASCs[50] (see Figure 1). The deletion of either TAF105 or OCA-B alone *in vivo* does not block Ig secretion[51,52]. Meanwhile, a set of enhancers [3' Igh (alpha) enhancers, HS1-4] are found far downstream of the whole *Igh* gene cluster with long range effects for heavy chain class switching and V-D-J recombination[53,54]. Our studies and those of others with transfected *Igh* genes showed full regulation of the secretory *vs* membrane alternative RNA processing choice with constructs that lacked the HS1-4 enhancers but retained the Emu/EH enhancer, see for example[55-58]. The Ig heavy chain EH/Emu enhancer stimulates transcription from functional promoters in B lymphocytes[53] but not other cell types.

The EH/Emu enhancer region most likely loops back to the promoter and communicates with it as shown in Figure 1; it contains binding sites for the indicated transcription factors. It appears that for squelching of lymphocyte-specific transcription in non-lymphoid cells, the binding of the repressive nuclear factor-μNR to the Igh enhancer prevents nuclear matrix attachment by interfering with the positively acting matrix attachment region proteins such as MAR-BP1, which drive transcription in B cells[59]. Igh 3' enhancer-bound OCA-B and promoter-bound TFII-I mediate promoter-enhancer interactions, in both cis and trans, that are important for Igh transcription. This suggests an important function for OCA-B in Igh 3' enhancer function *in vivo* that may be important for high levels of secretory-specific mRNA production[60].

THE UPBEAT: ELONGATION SETS THE TEMPO OF THE MARCH FOR IG SECRETION

Antibody molecules are first expressed on the surface of maturing B cells as membrane spanning receptors for immunogens and are known as the B cell receptor or BCR. Engagement of the BCR by cognate antigen on a mature B cell leads to activation of transcription and cell growth as described above. After the activation process, the pre-mRNA transcribed from the rearranged Ig heavy chain gene is alternatively processed to produce the secretory-specific form of Igh mRNA, reviewed in[4]. Not only is there a shift to use of the proximal poly(A) site but also a large increase in the overall amount of mRNA, with a less than 2-fold increase in RNAP-Ⅱ loading on the gene[61]. This indicates that RNA processing increases the quality and the quantity of the mRNA made from the Igh locus. The transition in Igh mRNA processing serves as a hallmark for the differentiation of the B cell to a plasma blast or an ASC and is a harbinger of major changes in cellular architecture and transcription allowing the Ig protein to be secreted[62]. The RNA polymerase in

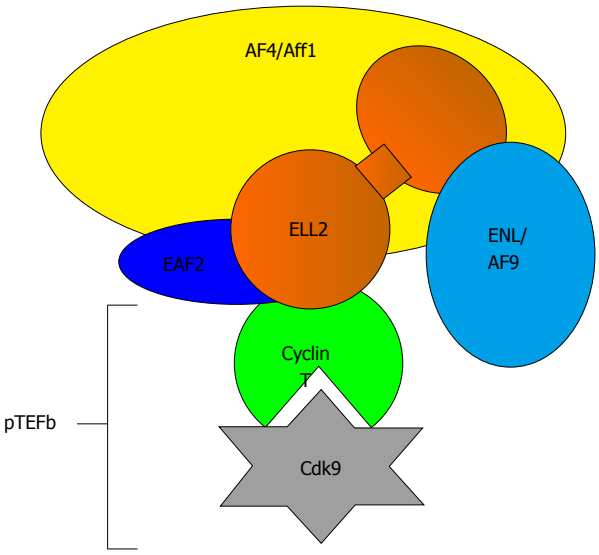

Figure 2 Components of the super elongation complex. ELL2 is part of the super elongation complex important for releasing RNA polymerase Ⅱ from pausing. Cdk9 phosphorylates the ser-2 of the heptad repeats in the carboxyl-terminal domain of RNAP-Ⅱ, negative elongation factor negative elongation factor, and DRB sensitive factor the DRB-sensitive factor, which becomes activated. ELL2: Eleven lysine rich leukemia protein 2; Cdk9: Cyclin dependent kinase 9.

the ASC is traveling to the beat of drum different from that in a B cell. Transcription elongation sets the tempo and acts as the drum major.

ELL factors equip RNAP-Ⅱ for elongation

All three members of the *ELL* gene family, 1, 2, 3, are involved in transcription elongation in the super elongation complex, SEC, see Figure 2. ELL1 was cloned from multiple lineage leukemia cells when its COOH terminal half was found to be a fusion partner with MLL, a histone H3 K4 methylase[63]. Based on the available literature, ELL1 may play its biggest role in DNA repair and small RNA synthesis[64,65]. Other family members were cloned because of their homology to ELL1. ELL3, at 397 amino acids long, differs in sequence from ELL1 and ELL2 (602 and 633 amino acids respectively) and lacks the central disordered region depicted in Figure 3, but retains the majority of the NH2-terminal productive elongation domain and the occludin homology/p53 interacting domain. Both ELL1 and ELL3 have been shown to sequester p53 and abrogate its activities[66,67]. ELL2 was not tested for this activity. ELL3 was first described as testis specific[68], but subsequently it was shown to play a role in the epithelial-mesenchymal transition[69] and to mark enhancers in ES cells, priming for future gene activation[70]. ELL2 replaces ELL3, which predominates in embryonic cells[69] and B cells; ELL3 levels are diminished after stimulation to ASC differentiation even in ELL2 conditional knockouts[62] see Figure 4. Its role in B cells is as yet undefined.

ELL1 was unable to substitute for ELL2 in driving proximal poly(A) site choice in the Igh locus[71]. ELL1 and 2 differ primarily in the sequence of the disordered region starting at amino acid 292 in Figure 3. Thus each ELL can be expected to have unique interactions and functions in transcription elongation based on its unique sequences. For example, Mediator subunit 26 drives the association of ELL1 with snRNA gene promoters[72]. Using yeast two-hybrid assays, we have shown that conserved portions of the central disordered, proline-rich regions of ELL1 and 2 bind specific proteins[73]; the absence of this region in ELL3 dictates that it will have different associations.

We have shown that ELL2 modifies the RNA polymerases in ASCs[10,61,71]; this causes RNAP-Ⅱ to traverse the genes in a manner that is unlike that in a B cell and hence the RNAP-Ⅱs in ASCs "travel to the beat of a different drum". ELL2 has important and now well established roles in releasing paused RNAP-Ⅱ in Human Immunodeficiency Virus (HIV) infection and in multiple myeloma[4,74]. There is a > 6-fold rise in the level of ELL2 ASCs (see Figure 4)[10,62,71,73,75], mediated by the Irf4 transcription factor[76-78]. There is also a increase in ELL2 mediated by Blimp-1 expression[76]. We showed that ELL2 drives alternative RNA processing [exon skipping and first poly(A) site choice] to influence the expression of the secretory-specific form of Igh mRNA at the expense of the membrane form[10] diagrammed in Figure 5A. This occurs because more mature mRNA results from every pass of the RNAP-Ⅱ; processivity is increased by ELL2. Studies of the ELL2 promoter (-1142 to + 154) show Irf4 and NF-κB p65 responsive sites[62], cyclic AMP response elements, and binding sites for the viral onco-protein Tax made in HTLV infection[79]. In the SEC, ELL2 associates with the positive transcription factor P-TEFb, AFF4, and other proteins found in fusions with MLL in cancer that facilitate H3K4 methylation[80], see Figure 2.

In the case of model ASCs *vs* B cells, more ELL2 and P-TEFb are recruited to the RNAP-Ⅱ on the identical *Igh* gene; there is a correspondingly higher level of ser-2 phosphorylation of the carboxyl-terminal domain (CTD) of RNAP-Ⅱ nearer the promoter[61,71]. The scope of modifications of the histones on the *Igh* gene is different from that seen in B cells and ASCs. We saw more H3K79 di- and tri-methylation as well as H3K4 methylation 3' of the internal heavy chain enhancer in ASCs, which is indicative of a more open chromatin configuration[71] (see Figure 5). H3K79 methylation has also been linked to alterations in splicing[81]. All of these changes in chromatin would favor use of promoter proximal poly(A) sites, like that of the secretory Igh poly(A) site and skipping of the splice sites that would be necessary for the production of the Igh membrane-encoding form of Igh. Ironically, an elongation factor causes the production of a shorter Igh mRNA.

Our B-cell specific ELL2 conditional knockout mice (ell2loxp/loxp CD19cre/+aka ELL2cKO)[62] exhibit normal numbers of splenic B cells but curtailed primary and secondary humoral responses both in NP-ficoll and NP-KLH immunized animals. In ELL2 cKO mice relative to ELL2+/+ animals: CD138+/ B220 lo ASCs in spleen were reduced; there were fewer IgG1+ antibody producing cells in the bone marrow (*i.e.,* long-

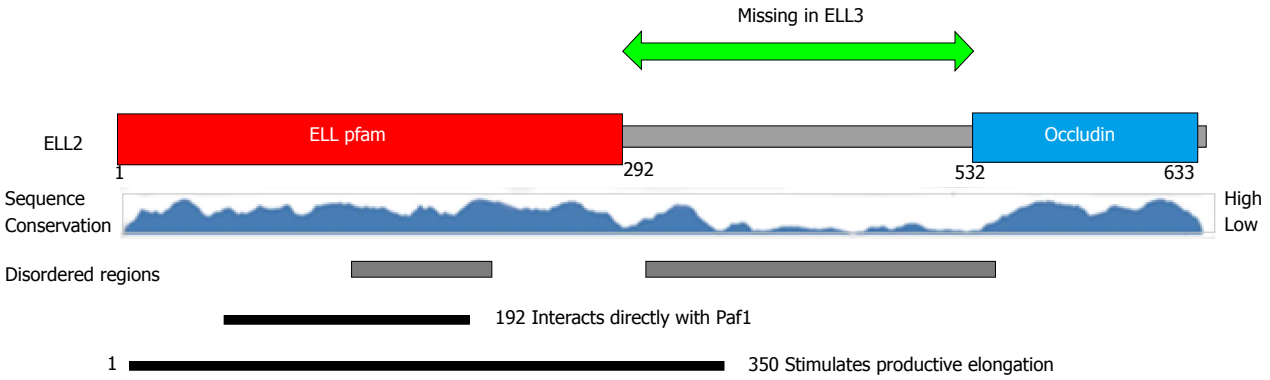

Figure 3 Protein structure of ELL2 *vs* ELL3. ELL2 contains three domains the common ELL protein family domain the disordered region and the occludin homology domain. The central disordered region is missing in ELL3 and the amino acid sequences vary in other regions as well. ELL: Eleven-nineteen lysine rich leukemia protein; pfam: Protein family; Paf1: Polymerase associated factor 1.

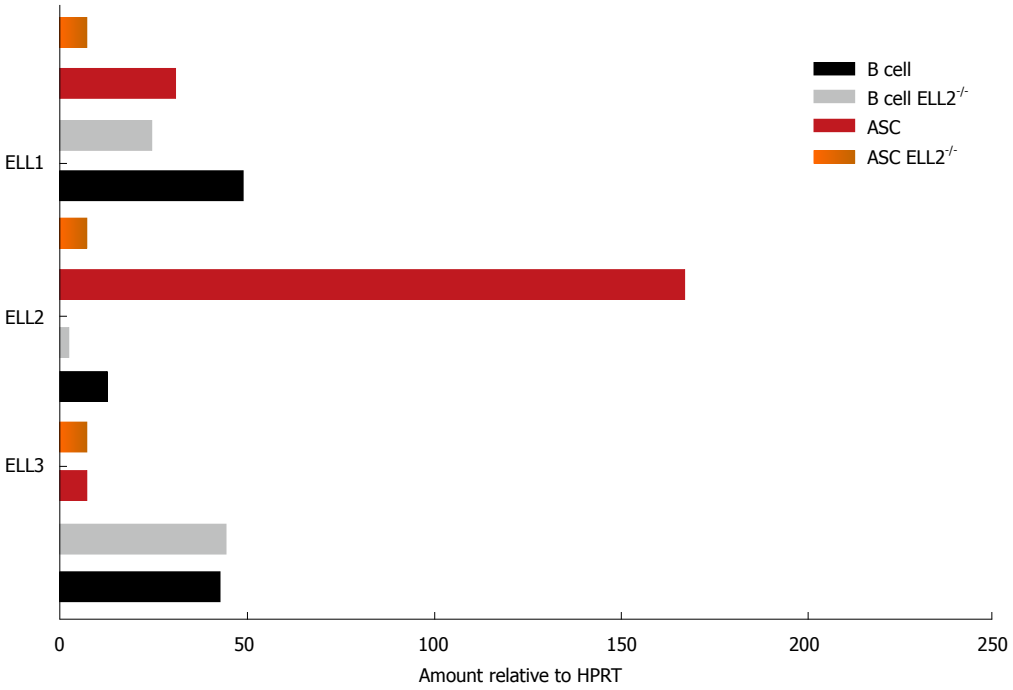

Figure 4 Expression of ELL1, 2 and 3 varies between B cells and antibody secreting cells. The expression of the mRNA for the three factors was measured by RT-QPCR relative to HPRT in both wild type mice and mice lacking ELL2 in their B cell compartment. HPRT: Hypoxanthine phosphoribosyltransferase; ASC: Antibody secreting cell; RT-QPCR: Reverse transcriptase quantitative polymerase chain reaction; ELL: Eleven-nineteen lysine rich leukemia protein.

lived plasma cells); splenic B cells stimulated by LPS *ex vivo* were ¼ as likely to produce B220loCD138+ cells (ASCs) than from control splenic B-cells. The "pseudo ASCs" that arise in the ELL2 cKO have a paucity of secreted Igh, and distended, abnormal appearing ER by electron microscopy. The amounts of Ig kappa, activating transcription factor 6 (Atf6), BCMA (Tnfrsf1), BiP, Cyclin B2, OCA-B, and Xbp1 mRNAs, unspliced and spliced, are severely reduced in the ELL2 cKOs[62]. Thus we showed that ELL2 is essential for antibody synthesis and export.

The complex expression pattern of the three ELL family members in B cells and ASCs both in ELL2$^{+/+}$ and the ELL2$^{-/-}$ conditional knockouts is shown in Figure 4. The knockout of ELL2 influences its own and ELL1 mRNAs but not that of ELL3, which declines following LPS stimulation to ASCs regardless of the presence of ELL2[62].

The super elongation complex acts at active genes

A combination of genome-wide high-throughput sequencing methods and drug treatments that inhibit P-TEFb have suggested that P-TEFb-driven release of paused RNAP-Ⅱ from promoter-proximal regions to begin productive elongation is a widespread and necessary step in transcription. Studies have shown that inhibition of P-TEFb, and by extension SEC, that prevents RNAP-Ⅱ release, blocks almost all transcription[9,29,30]. Thus, all active genes experience a potentially rate-limiting pausing step in the transcription cycle and require SEC activity for gene body transcription. However, this pause step causes a significant accumulation of promoter-

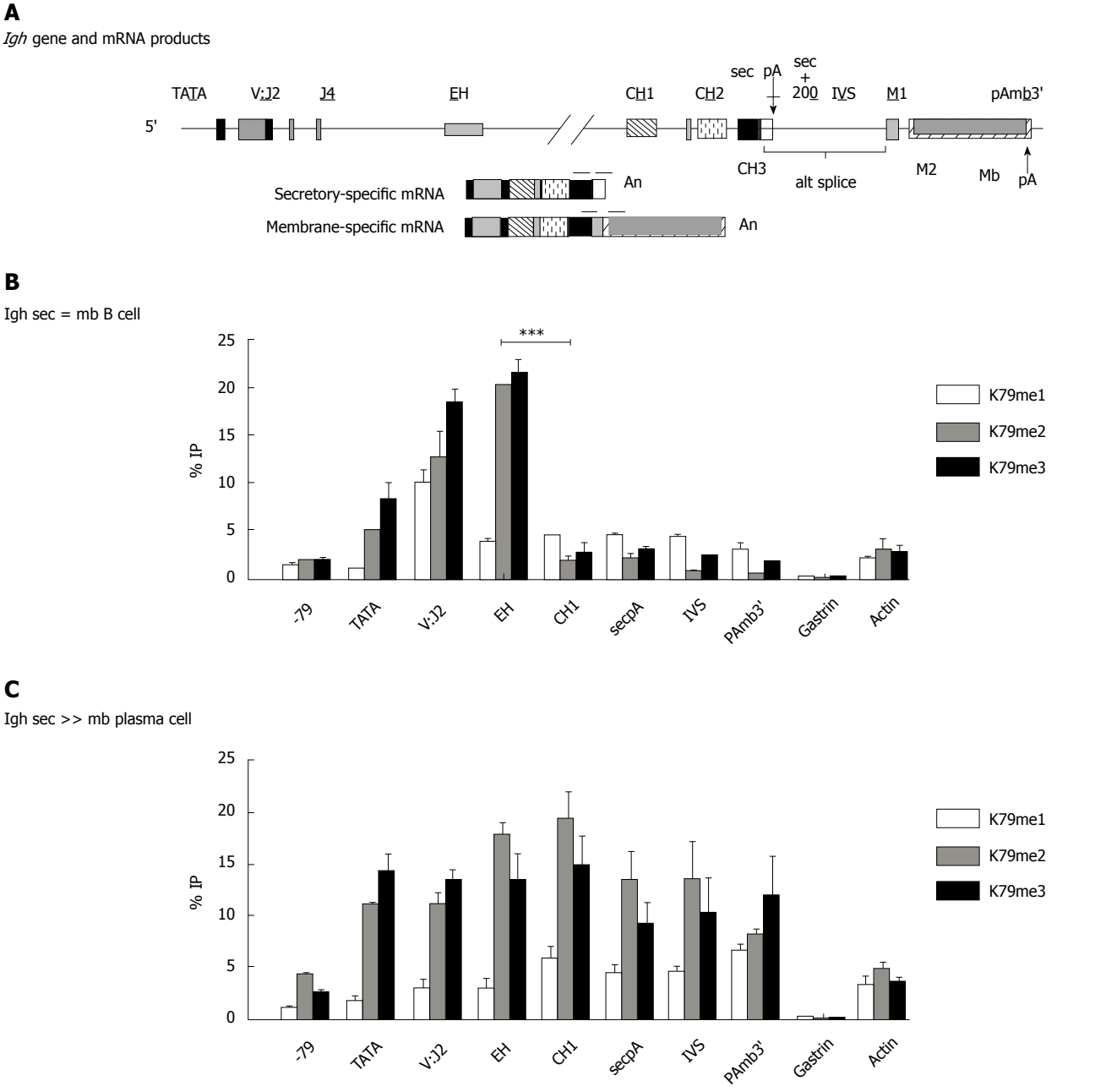

Figure 5 ***Igh*** **gene has a different pattern of H3K7methylation in B cells** ***vs*** **antibody secreting cells.** Distribution of histone H3K7me is enhanced in the region downstream of the internal Igh enhancer in plasma cells (ASCs). The 11 kb *Igh gamma 2a* gene is identical in the B and PC (hybridoma) lines and located in the intact Igh locus. A: Location of probes used in QPCRs. Cells were fixed and chromatin IP performed with the indicated antibodies specific to the individual K79 methylations; B: The B cell line A20; C: The plasma/hybridoma line AxJ which is an ASC. ASCs: Antibody secreting cells; QPCR: Quantitiative polymerase chain reaction; IP: Immunoprecipitation.

proximally paused RNAP-Ⅱ only at a subset of active genes in untreated cells (40%-70%, depending on the method and cell type)[3,9,29,31-33]. Presumably SEC activity is simply not limiting on the remainder of genes that do not show accumulation of paused RNAP-Ⅱ. This finding indicates that a pausing- and SEC-dependent release step could become a rate-limiting and potentially regulatory step at all active genes[82].

Transcription and RNA processing are controlled in cells by the cooperating processes of modifications to the CTD of RNAP-Ⅱ, addition of elongation and RNA processing factors to the RNAP-Ⅱ complex, and by chromatin modifications[83]. Negative elongation factor (NELF) and DRB sensitive factor (DSIF) are recruited to an RNAP-Ⅱ when it pauses just after initiation of transcription. DSIF is composed of the highly conserved Spt4 and Spt5 subunits, which have been shown to have unique parts to play at different phases of Ig class switch recombination[84] and in germinal center B cells[85]. NELF is found only in paused metazoan, not yeast transcription complexes[86]. Recruitment to the paused RNAP-Ⅱ of P-TEFb, composed of cyclin T and cdk9, and its associated factors like ELL into a super elongation complex[87], results in phosphorylation of DSIF and the

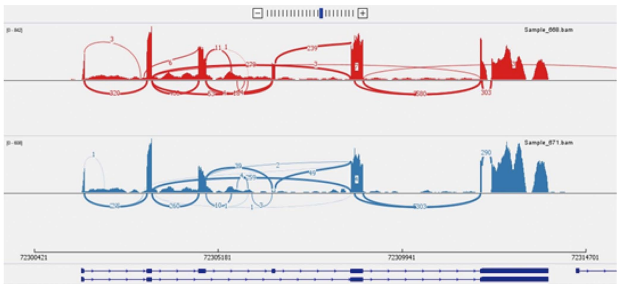

Figure 6 Sashimi plot depicting exon skipping. Xaf1 Sashimi plot obtained from RNA-Seq of ELL2 WT (red track) and cKO (blue track) antibody secreting cell samples. This plot demonstrates the exon skipping of exon 4 occurring in the cKO. The arcs indicate splice junction reads, with the thickness of the arc correlating with the number of junction reads spanning the two exons being connected by the arc. cKO: Conditional knockout; RNA-seq: RNA sequencing; WT: Wild type.

ser-2 of the carboxyl-terminal end of RNAP-Ⅱ. NELF is also phosphorylated by P-TEFb, releasing it from the now elongation-competent RNAP-Ⅱ complex. It is clear from studies with HIV tat and tar that recruitment of P-TEFb with ELL2 also facilitates interactions of the five-subunit polymerase associated factor (paf) with RNAP-Ⅱ; paf then recruits the polyadenylation factors[88]. This would favor promoter proximal polyadenylation. Studies using RNAP-Ⅱ mutants or drugs to slow elongation show that reduced transcription rates are coupled with alternative exon inclusion while speeding up the polymerase causes exon skipping[89]. In addition, transcription factors have been shown to control the use of alternative exons and control splicing patterns, presumably by differentially setting up the RNAP-Ⅱ complex or its elongation rate[90].

Effects of altered RNAP-Ⅱ on other genes

Using deep mRNA sequencing, the knockdown of ELL2 by siRNA in a plasma cell line was shown to influence several other genes besides Igh secretory specific mRNA processing, namely several splicing factors, cyclin B2 (Ccnb2), and the B cell maturation antigen (Tnfrsf17) aka BCMA. Long term survival of plasma cells is impaired by the lack of BCMA in a knockout mouse[91]. But loss of BCMA alone in -/- mice does not alter humoral responses (T-independent or T-dependent) nor the formation of short-lived plasma cells, yet loss of ELL2 in mice does[62]. Benson *et al*[92] saw changes in splicing in a number of genes involved in mRNA processing; this would have had other far reaching secondary effects beyond that of ELL2 on transcription. Dissociating the direct *vs* indirect effects of ELL2 is key to understanding its role in changing the RNAP-Ⅱ and RNA processing patterns on a given gene in ASCs. In the ELL2 conditional knockout mice we saw not only reduced Igh processing to the secretory-specific form, but also deficiency in light chain mRNA synthesis and decreased expression of *UPR* genes, especially in Xbp1. We also saw changes in the splicing of some ELL2 target genes as illustrated in the Sashimi plot shown in Figure 6 for *Xaf1*, a gene involved in apoptosis of cells[93].

Elongation factors like ELL2 not only change RNA processing patterns but they also increase the processivity of RNAP-Ⅱ[94]. As a consequence, they can cause higher production of mature mRNA from a precursor and boost mRNA yields without increasing RNAP-Ⅱ loading. For example, using a cyclin B2 promoter, addition of ELL2 cDNA had a greater than 7-fold enhancement in the luciferase reporter systems relative to a Blimp-1 promoter[62]. It is also worth noting that many of the genes in primary B cells affected by the loss of ELL2 after stimulation to ASCs are genes expressed at high mRNA abundance (Atf6, BiP, Igh, IgL, Pou2af1, Xbp1), genes for which efficient pre-mRNA to mature mRNA processing would be important. Interestingly, in the ELL2 knockout, there was a 5-fold decrease in the expression of Pou2af1 (OBF-1/ BOB-1/OCA-B) mRNA, a putative downstream target of Xbp1[95]. We also saw that ELL2 could enhance luciferase yields from a promoter carrying the UPR elements, so the effect on Pou2af1 could be direct as well as indirect through decreased Xbp1.

THE UPR: SUPPLYING THE BACK BEAT FOR IG SECRETION

The increase of ELL2 in ASCs drives alternative RNA processing and leads to an increase in secretory Igh mRNA[10]. The substantial amount of Ig chains being produced must first be processed efficiently into antibodies by the ER. Differentiating B cells adapt to the added stress of processing the increased amount of antibody by inducing the UPR, a signaling cascade prompted by ER stress that upregulates ER chaperone and folding enzymes expression (reviewed in[96]). Even after initiation of terminal B cell differentiation and efficient elongation take place, regulators of the UPR and alleviators of ER stress are needed to ensure that successful ASC development occurs.

Xbp1 regulates UPR for ASC differentiation

In mature ASCs, the ER response is unique from that seen in other cells[97]. The UPR in many cells typically has three arms, the Ire1/Xbp1 pathway, an Atf6 pathway, and the PERK pathway[98]. But PERK knockout mice secrete normal amounts of Ig, while PERK protein expression is not changed significantly between B cells and ASCs[99,100]. In addition, Atf6 is not necessary for the development of ASCs; thus when B cells are stimulated to secrete antibody, the primary pathway for ER remodeling appears to reside in the Ire1 to Xbp1 pathway[101].

Aggregation and then auto phosphorylation of Ire1 causes it to acquire the ability to specifically cleave and then splice Xbp1 mRNA; the newly spliced Xbp1 RNA species encodes a novel Xbp1 protein with transcriptional activity on its own promoter and other UPR promoters containing the UPR element UPRE[99]. In an Xbp1 conditional deletion, the mice show defects in ASC development[25] and low levels of secretory Ig[102]. But it has been argued that the consequences of Xbp1 deletion alone are relatively mild[103]. ASCs are

present in normal frequencies in resting and immunized animals, and Ig secretion is reduced but not eliminated in conditional Xbp1 knockouts. Thus the gene regulatory program controlling ASC differentiation may proceed relatively normally in the absence of Xbp1[103].

On further analysis, the low levels of Igh mRNA in Xbp1$^{-/-}$ mice result from the 8-fold increased levels of Ire1-P over control; the highly abundant Ire1-P cleaves the Igh mu secretory mRNA[104]. This is a process similar to the previously described pathway[105] in which Ire1-P can act to cleave its own mRNA, as well as other RNAs in a process called regulated IRE1-dependent decay[106]. Only Xbp1 mRNA is spliced, not cleaved, by Ire1-P to form a new functional RNA[107]. A double deletion of Xbp1 and Ire1 restores IgM secretion by inhibiting Ig mRNA degradation[104]. Mutations in the Ire1 nuclease function cause only a 2-fold reduction in Ig secretion[108]. Taken together, this leads to a conclusion that some Ig secretion can occur without the unusual cleavage and splicing of Xbp1 and there may be other proteins that allow for the upregulation of the UPR besides the spliced mRNA encoded Xbp1. As we discussed above, ELL2 has a role in enhancing the transcription of other UPR proteins through the UPR element[62] thereby linking production of the Igh secretory mRNA and the build-up of the UPR. Activation of the mammalian target of rapamycin (mTOR) pathway can also bypass Xbp1 for Ig secretion[109].

mTOR bypasses Xbp1 for ASC differentiation and Ig secretion

mTOR is a vital serine/threonine kinase with two known subunit complexes, mTORC1 and mTORC2[110]. Much of the known function of mTOR, primarily in complex one, shows major roles in cellular proliferation[111] and Ig secretion[112]. The main function of mTORC1 is to recognize nutrient levels and mitogenic signals, and with these trigger cellular growth and proliferation. mTORC2 differs as it is nutrient independent and is activated by growth factors[113]. Within its pathway is the tuber sclerosis complex (TSC), which is an inhibitory complex of mTOR. TSC presents itself in two forms, TSC1 and TSC2. The two forms come together as a heterodimeric complex[114,115]. Akt, a protein kinase, is responsible for phosphorylation of TSC2 and is activated after LPS-stimulation[115]. The release of TSC complex inhibition induces mTOR *via* BCR stimulation. The reversion of TSC1 inhibition of mTORC1 is responsible for protein synthesis in LPS-activated B cells, which is coupled with substantial ER stress[116]. ER stress can activate the UPR, restoring ER stability, or possibly lead to autophagy or apoptosis[117]. During B cell differentiation to ASC, ER remodeling is substantial and exclusively facilitated by the Xbp1 UPR pathway. Skipping of the Xbp1 pathway has been shown to allow B cells to viably differentiate into long-lived ASCs that only secrete small amounts of Igs[103,104]. It was shown that the ER morphology was highly compromised in the Xbp1 knockout after ASC transition. The successful transition, even with compromised ER morphology, from B cell to ASC was shown to be due to the positive regulation of mTOR[109].

Looking directly at mTOR, inhibition of mTORC1 in mice induces macro-autophagy[118]. When activated, mTORC1 promotes cell growth and protein synthesis. Along with mTORC1, TSC1 ablation *ex vivo* resulted in cell death of developing ASCs[116]. The production of a TSC1 KO, Xbp1 KO and TSC1/Xbp1 DKO has allowed for complete analysis of mTORC1 outcomes independent of parallel pathways. TSC1 KO promoted ASC differentiation with increased mTOR activity along with an unexpected increase in Ire1. Pertaining to the antibody secretion of the ASCs, the expected reduction of IgM and IgG1 levels in the KOs and DKO was observed. A marked increase of IgA titers in the serum of the DKO was also observed. This correlates with the novel finding that mTOR activation can bypass Xbp1 for antibody secretion. To assess the effect of TSC1 KO on the ER, the DKO and the Xbp1 KO were compared after LPS stimulation. It was clear that the double knockout had a less compromised ER, suggesting that mTOR activation and its directed UPR play a crucial role in ER maintenance and remodeling[109].

CONCLUSION

The modification of RNAP-Ⅱ elongation by ELL2 in ASCs is dramatic with far-reaching consequences. It is important to further study the direct effects that this modification is having on transcription of Igh and on expression of other *ASC* genes. What else occurs as a result of RNA polymerases traveling to the beat of a different drum? The significance of understanding these systems lies in the foundation of the correct production and processing of antibodies, a vital part of immune response. Further study will allow for an expanded breadth of understanding concerning this complex system as well as great advances in diagnosis and therapy for autoimmunity and immune-deficiency diseases.

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REVIEW

Dendritic cells and the extracellular matrix: A challenge for maintaining tolerance/homeostasis

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Abstract

The importance of the extracellular matrix (ECM) in contributing to structural, mechanical, functional and tissue-specific features in the body is well appreciated. While the ECM was previously considered to be a passive bystander, it is now evident that it plays active, dynamic and flexible roles in shaping cell survival, differentiation, migration and death to varying extents depending on the specific site in the body. Dendritic cells (DCs) are recognized as potent antigen presenting cells present in many tissues and in blood, continuously scrutinizing the microenvironment for antigens and mounting local and systemic host responses against harmful agents. DCs also play pivotal roles in maintaining homeostasis to harmless self-antigens, critical for preventing autoimmunity. What is less understood are the complex interactions between DCs and the ECM in maintaining this balance between steady-state tissue residence and DC activation during inflammation. DCs are finely tuned to inflammation-induced variations in fragment length, accessible epitopes and post-translational modifications of individual ECM components and correspondingly interpret these changes appropriately by adjusting their profiles of cognate binding receptors and downstream immune activation. The successful design and composition of novel ECM-based mimetics in regenerative medicine and other applications rely on our improved understanding of DC-ECM interplay in homeostasis and the challenges involved in maintaining it.

Key words: Dendritic cells; Extracellular matrix; Tolerance; Biomaterials; Homeostasis; Regenerative medicine;

Biointeractive implants

Core tip: The extracellular matrix (ECM) provides an essential framework for tissues in the body as well as actively orchestrates diverse cellular functions. Professional antigen presenting cells namely dendritic cells (DCs) are uniquely positioned to distinguish between self and non-self and accordingly regulate systemic immunity or tolerance. DCs and the ECM participate in finely-tuned, dynamic exchanges that ultimately impact the equilibrium between steady-state DC tissue residence or DC-instigated inflammation. To design biointeractive, ECM-inspired implants for regenerative medicine applications that retain functionality and undergo successful integration long-term, it is critical to understand the challenges involved in maintaining DC-ECM immune homeostasis under normal conditions.

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INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that differentiate self from non-self and play crucial roles in determining the balance between tolerance and immunity. They were first discovered in mouse spleen by Steinman and Cohn[1] in a seminal paper published in 1973. Since this initial finding, the dogma that DC maturation is essential for the initiation of immunity has been well established. Immature DCs are typically distinguished by high levels of intracellular major histocompatibility complex class II (MHCII), low expression of adhesive and costimulatory receptors, strong endocytic, pinocytic and phagocytic abilities and weak capacities for T cell stimulation[2,3]. Immature DCs mount an immune surveillance program mediated in part by extracellular or cytoplasmic pattern recognition receptors (PRRs) expressed by DCs that identify evolutionarily conserved pathogen associated molecular pattern motifs on bacteria, viruses and on other foreign bodies or endogenous damage-associated molecular patterns (DAMPs). These recognition events trigger their transformation from rounded immature DCs into terminally differentiated mature DCs with enhanced motility, that exhibit extended dendritic processes and upregulate expression of co-stimulatory (CD80, CD86), MHCII molecules and adhesion molecules which bind T cells[2]. Ingested antigens processed intracellularly and presented as spliced peptides are loaded on to MHC complexes on mature DCs. The interactions between MHC and T cell receptors [MHCI: cytotoxic T cells (T_C) and MHCII: helper T cell (T_H) subsets] expressed on T cell surfaces drive antigen-specific cellular immunity. Notably, DCs are unique in their capacity to trigger T cell immunity. Taken together, DC-driven generation of cytokines, chemokines and other factors together orchestrate downstream host protective antigen-specific adaptive immune responses, as reviewed in[2]. DCs have three broad functions - mounting an immune response, maintaining immune tolerance and regulating immune memory, underscoring their critical roles in maintaining the balance between immunity and homeostasis[2].

This review discusses the growing body of evidence that the extracellular matrix (ECM) orchestrates DC interactions at different sites both in homeostasis and in inflammation. These interactions are highly complex and have many redundancies since DCs possess numerous receptors capable of binding the multicomponent ECM and are capable of upregulating and downregulating the expressions of PRRs in response to alterations in the ECM, thereby making it essential to better understand this interchange. Many questions remain incompletely elucidated: Why DCs are selective in terms of residing in certain tissues but not in others. For instance, what roles do tissue specific cytokines, chemokines and other factors associated with and released by the ECM play in regulating DC behaviour? To what extent are other tissue resident cells responsible for maintaining DC homeostasis, *e.g.*, hepatic stellate cells (HSCs) in the liver[4], epithelial cells in intestine[5], or keratinocytes in skin[6]. Finally, how do alterations in the ECM affect the DC steady-state and are these alterations reversible?

DC SUBSETS

Over the last few decades several groups have contributed to the large body of work performed in characterizing DCs present in tissues and in blood and their *in vitro* generated counterparts. DCs are identified by the expression of MHCII and costimulatory markers, and the absence of lineage markers such as CD3 (T cell), CD14 (monocyte), CD19 (B cell), CD56 (natural killer cell) or CD66b (granulocyte)[2]. DCs are described as two types: Conventional DCs (cDCs) and plasmacytoid DCs (pDCs). The cDCs are a principal DC subset and are sub-divided into migratory and non-migratory. Migratory DCs arise from the tissues and reach secondary lymphoid organs (SLO) *via* the lymphatics. Tissue resident conventional migratory DCs are broadly classified as $CD103^+$ $CD11b^-$ and $CD11b^+$ cDCs and have been described in intestine, liver, lung, kidney and skin as reviewed in[7,8]. The specific locations and roles of the different DC subsets vary in different tissues. For instance, lung DC subsets include migratory $CD103^+$ $CD11c^{high}$ $CD11b^-$ DCs in the intra-epithelial network, $CD103^-$ $CD11c^{high}$ $CD11b^+$ DCs in the lamina propria (LP) and non-conventional pDCs[8]. Intestinal DCs in Peyer's Patches (PP), LP and mesenteric lymph nodes exhibit varied expression of CD103 and CX3CR1 of which migratory, conventional $CD103^+$ DCs have been

assigned both immunogenic and tolerogenic roles[8]. Liver DCs consist of $CD11c^+$ $B220^-$ DCs (further divided into $CD103^+$ and $CD103^-$), both linked to regulatory T cells (T_{reg}) induction as reviewed in[8] and $CD11c^+$ $B220^+$ pDCs, the latter playing a role in maintaining steady state tolerance and the resolution of inflammation after liver injury[8]. Kidney DCs comprise $CX3CR1^+$ $CD11b^+$ DCs, $CX3CR1^+$ $CD11b^-$ DCs and $CD103^+$ DCs, while $CD103^+$ cells are thought to play a tolerogenic role as opposed to other subsets as reviewed in[8]. Skin DCs include dermal DCs which are Langerin+ ($CD207^+$) $CD103^+$ DCs and are categorized as cDCs with bone marrow precursors, as well as Langerin- $CD103^-$ DCs (with unknown precursors)[8].

Conventional DCs also include lymphoid DCs that are distinct from myeloid DCs, lack expression of CD11b, CD13, CD14, and CD33 and are derived from precursors that have the ability to differentiate into T cells and NK cells as reported in[3] as opposed to monocyte/macrophage lineages[9]. While cDCs sample tissue antigens and migrate to present the processed peptide(s) to T cells in LNs, in contrast, non-migratory cDCs previously considered as "lymphoid" DCs reside in thymus, spleen, LNs or PP[3,10]. Lymphoid DCs include $CD4^-CD8\alpha^+$ DCs that cross present antigen to $CD8^+$T cells, while $CD4^+CD8\alpha^-$ DCs in spleen or $CD4^-CD8\alpha^-$ DCs in mucosal associated lymphoid tissue, activate $CD4^+$ T cells. Non-migratory lymphoid DCs regulate thymic negative selection, drive T_H2 responses in humans and stimulate regulatory responses overall[3].

The pDCs are found mostly in lymphoid tissues and secrete IFN-α upon exposure to viral antigens. Phenotypically, pDCs, the second principal DC subset express CD45RA, CD123, CD303 and CD304 as well as low levels of MHCII, costimulatory molecules and CD11c, while myeloid DCs express CD11c, CD13, CD33 and CD11b[11]. The pDCs are highly secretory, exhibit plasma cell-like morphologies and display properties of both cDCs and lymphocytes. Importantly, pDCs express endosomal TLR7 and TLR9 that detect viral single stranded RNA and unmethylated CpG-containing DNA and respond by rapid and substantial production of type Ⅰ IFN (IFN-α/β)[12]. Plasmacytoid DCs start in bone marrow and enter lymphoid tissues where they mainly reside, through blood[7]. Plasmacytoid DCs are important for mediating differentiation of B cells to plasma cells for antibody production and have been linked to immunogenic and tolerogenic responses in the liver and lung as reviewed in[8]. Most DCs [apart from yolk sac derived - Langerhans cells (LCs)] are generated in bone marrow from myeloid progenitor cells[13] with some *in situ* proliferation in spleen. Another class of non-conventional DCs namely monocyte-derived "inflammatory" DCs have been detected in the skin and kidneys and intestine and have been implicated in the progression of inflammation in colitis and as $CD103^-$ $CX3CR1^+$ DCs in maintaining gut homeostasis. In the lung, $CD103^-$ $CD11c^{high}$ $CD11b^+$ DCs play crucial roles in reacting to allergens and triggering T_H2-mediated immunity. Finally, self-renewing DC-like cells such as slow turnover LCs and microglia are specialized dendritiform cells derived from the yolk sac and reside in the squamous epithelium and in CNS parenchyma respectively and mediate tolerance in the resting states[8,11].

It is clear that DCs are not narrowly defined as a single type of cell but instead represent a diverse assortment of cells derived from different lineages[10,14-17]. The generally accepted theory is that hematopoietic DC progenitors from the bone marrow circulate through the body and are receptive to specific combinations of cytokines and signals, resulting in DC subsets with specialized homing properties and roles. *In vitro* DCs have been generated from $CD14^+$ monocytes in blood and $CD34^+$ bone marrow precursors[2]. "Classical" myeloid DCs have been generated from myeloid committed $CD34^+$ progenitor cells and monocytes treated with granulocyte macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor-α (TNF-α) ± interleukin-4 (IL-4) *in vitro*[7,8,11,18,19]. Myeloid DCs regulate responses of CD4 and CD8 T cells and are involved in B cell differentiation into plasma cells. In addition, $CD34^+$, $CD14^-$ cells differentiate into LCs in the presence of transforming growth factor-β (TGF-β)[7,8,11,18,19]. Also, lymphoid committed $CD34^+$ cells become pDCs in the presence of IL-3[20]. Myeloid DCs are sometimes referred to as DC1 and express toll-like receptor 2 (TLR2), TLR3, TLR4, TLR7 and activate naïve T cells along T_H1, T_H2 pathways[7,8,11,18,19]. In contrast, lymphoid DCs or DC2 express TLR7 and TLR 9 and secrete IFN-α in response to invading viruses. Notably, high numbers of DCs have been generated in mice and in humans by recurrent injections with hematopoietin flt-3L, thought to act on DC precursors in the bone marrow[2,21,22].

Interestingly however, when tissue residence is discussed there is little reference to how the tissue might affect or even permit the DCs to migrate into tissue matrices and egress from it *via* the lymphatics. Furthermore, changes in the tissue which occur during inflammation will affect not only the resident DCs but also newly recruited DCs. For instance, the retina in normal mice has a small population of $MHCII^+$ $33D1^+$ DCs located mainly at the periphery while tissue resident microglia are macrophage-like cells. However, during inflammation, *e.g.*, uveoretinitis, there is a marked increase in the numbers of antigen-presenting cDCs[23]. Microglia mostly maintain tolerance in non-inflamed retina but can become activated during degenerative disease such as age-related macular degeneration (AMD) or inherited retinal degeneration[24]. Dysregulated clearance/accumulation of debris result in microglial activation accompanied by elevated production of pro-inflammatory chemokines and cytokines. Similarly, bone and cartilage do not have DCs under steady state conditions[25], although activated DCs can trigger cartilage degradation by producing TNF-α. It is not clear why certain tissues restrict DCs from being present in steady state or why others permit their entry. It would

be fascinating to gain an understanding of what the microenvironmental cues provided by the ECM towards this are as well as how these signals are altered in pathological conditions to pave the way for DC infiltration and subsequent immune responses.

FUNCTIONAL DICHOTOMY OF ECM

It has been well established that the ECM plays critical roles in regulating cellular differentiation, survival, shape and function including adhesion, motility, apoptosis and tissue specific alignment[26,27]. The ECM is a three dimensional mixture of triple helical collagens, complex proteoglycans composed of glycosaminoglycans covalently linked to protein, glycoproteins, proteases, growth factors and cytokines that respond actively to microenvironmental conditions[28,29]. Notably, dysregulation or mutations in the ECM have been linked to developmental, degenerative, malignant, and pathological states such as cancer and inflammatory arthritis[26], while oxidative impairment of ECM components by enzymatic or non-enzymatic pathways has been associated with progression of kidney disease, lung disease, arthritis, and chronic inflammation[30]. Protein fragmentation has been proposed to form site-specific focusses for free radicals and reactive species as suggested in this review[31].

Remarkably, the ECM displays functional dichotomy. Besides forming a supporting mesh to stabilize cells, the ECM plays active roles in regulating normal or pathological states of inflammatory cells[27]. Degradation of intact steady state high molecular weight proteins to low molecular weight fragments has been directly linked to initiating and contributing to the progression of inflammation as demonstrated for major constituents of the ECM such as collagen, elastin, laminin, hyaluronan (HA) or fibronectin, based on their effects on neutrophils, monocytes, macrophages[27,32-34]. In chronic lung neutrophil-mediated diseases that affect the matrix such as chronic obstructive pulmonary disease or in cystic fibrosis, evidence suggests that the products of protease degradation of matrix proteins (elastin, collagen fragments) are active triggers of inflammation (chemotactic for neutrophils)[35]. Interestingly, the degradation of interstitial matrix components such as collagen can induce peripheral blood mononuclear cells (PBMC) activation *via* IL-1β production, and to different extents depending on the nature of the collagen peptide[33]. In homeostasis, matrix components such as fibronectin play essential roles in mediating tissue cell adhesion and stabilize the ECM by interactions with fibrinogen. In contrast, fibronectin fragments detected in synovial fluid in rheumatoid arthritis (RA) display pro-inflammatory characteristics such as enhanced monocyte chemoattraction, phagocytosis of polymorphonuclear leukocytes and complement engagement as compared to intact fibronectin[34]. Similarly, ECM components can play dual roles, one during cardiac development and second in healthy recovery or persistent heart failure after myocardial infarction as indicated by the diverse expression of ECM factors in different physiological states (foetal, neonatal, adult)[28,36]. Importantly, ECM interactions with cytokines including fibroblast growth factor, TGF-β, interferon-γ (IFN-γ), macrophage inflammatory protein-1β, ILs or TNF-α and enzymes such as heparanase, urokinase-type plasminogen activator, elastase or matrix metalloproteinases (MMP) regulate the increase or decrease of inflammation at sites of tissue injury[37]. Blocking heparanase activity helps counteract early ECM degradation by controlling early inflammation[29]. Heparan sulfate in ECM binds chemokines and cytokines such as IL-2 and IFN-γ in steady state and when freely available, IL-2 triggers immune responses underscoring the point that ECM channels the host response towards or away from homeostasis[29]. Taken together, the picture emerging is that contrary to earlier concepts, the ECM is not a passive bystander but actively participates in the overall immune response. Indeed, the notion that the tissue regulates the immune response has been proposed by Matzinger[38] although precisely how awaits discovery. At the microenvironmental level, the matrix is dynamic and attuned to the stage of the immune/inflammatory response, prompting factors that are pro-inflammatory early on in the response to have anti-inflammatory effects during wound resolution[29].

TISSUE MATRICES AND DCS IN HOMEOSTASIS

DCs occupy diverse matrices in different tissues. The matrices assist in preserving DC tolerance through unique interactions and support their immune surveillance program. Some of these examples have been briefly reviewed here namely for skin, intestine, liver, retina, cornea and spleen. Although this is not a comprehensive list, immune privilege at different sites has been exhaustively reviewed elsewhere[39]. Long-lived LCs reside in skin epidermis (Figure 1) and maintain homeostasis by forming E-cadherin junctions with keratinocytes and by TGF-β mediated events *via* suppression of pro-inflammatory factors IL-1, and TNF-α[6,40,41]. Resting epidermal LCs in normal adult human skin importantly have the capacity to preserve immune homeostasis by stimulating tolerogenic skin resident T_{reg} responses to self-antigens and can also elicit activation of T_{eff} cells in response to foreign pathogens[42]. Interestingly, $CD1a^+$ and Birbeck granule expressing LCs also express neuronal receptors and communications between LCs and nerves suggest bidirectional signalling towards sustaining homeostasis[43]. Homeostasis and development of specialized DCs such as LCs and microglia rely on IL-34 secreted by epidermal keratinocytes and brain neurons respectively[44] Neuropeptides have been shown capable of regulating DC function. Interestingly, the neurotransmitter neurokinin A activates bone marrow-derived DCs to drive type 1 immune responses by targeting the neurokinin-2 receptor on

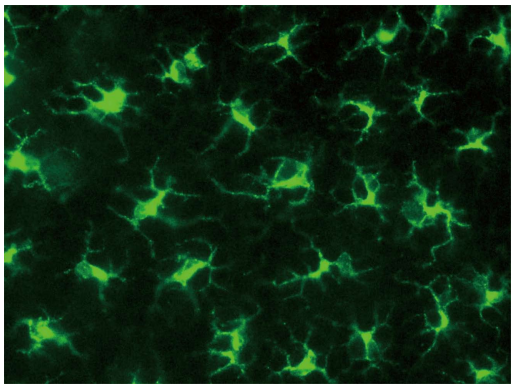


Figure 1 Murine Langerhans cells in ear skin reside in the epidermis, stained here for major histocompatibility complex class Ⅱ (green). Reprinted from Jakob T, Ring J, Udey MC. Multistep navigation of Langerhans/dendritic cells in and out of the skin. *J Allergy Clin Immunol* 2001; 108: 688-696 Copyright (2001), with permission from Elsevier.

DCs[45], while other neuropeptides such as Substance P, calcitonin gene-related peptide (CGRP) and somatostatin importantly can be secreted by DCs and regulate T cell activation[46]. Further, signalling *via* type 1 CGRP receptor on human DCs downregulates expression of MHCII and CD86 as well as decreases DC-associated T cell proliferation[47].

Similar to the skin, intestinal mucosa represent large surface areas exposed to the outside environment. It is therefore critical to maintain DC tolerance under steady state conditions. Intestinal $CD103^+$ $CD11b^+$ migratory DCs in LP, PP, gut-associated lymphoid tissues or solitary intestinal lymphoid tissue are key regulators of homeostasis[48] and are capable of inducing anti-inflammatory T_{reg} differentiation[5]. Also, $CX3CR1^+$ and $CD103^+$ mucosal DCs in the LP are important in maintaining gut immune homeostasis[49]. Furthermore, DCs in PP and mucosal DCs in LP are involved in generating oral tolerance to collagen Ⅱ in collagen-induced arthritis models, mediated by TGF-β, T_{regs} and tipping the balance towards T_H2 cytokines (IL-4)[50].

Liver resident professional and non-professional APCs including Kupffer cells (KCs), liver sinusoidal endothelial cells and DCs are crucial in maintaining hepatic tolerance under non-inflammatory conditions[51,52]. The HSCs located in perisinusoidal spaces of the liver have the ability to present antigen under tolerogenic conditions and exhibit cytoplasmic interactions with a broad range of functionally diverse cells such as hepatocytes, sinusoidal endothelial cells and KCs[4]. Specifically, HSCs coexist with murine liver DCs *in vivo* under homeostatic conditions and were shown to downregulate DC activation *via* tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase expression, towards establishing an anti-inflammatory phenotype[4]. Specifically, resident immature DCs operating in the microenvironment of anti-inflammatory IL-10 and TGF-β are tolerogenic and block the activation of liver penetrating lymphocytes *via* interactions of cytotoxic T lymphocyte associated antigen receptor-4 and PD-1, both of which are potent negative regulators of T cells[51].

The transparent, avascular cornea at the anterior of the eye is comprised of the epithelium, the highly stratified layers of collagen types Ⅰ and Ⅲ that form the stroma and the innermost endothelial layer. Towards sustaining homeostasis in the normal healthy state, $CD11b^+$ $CD11c^+$ DCs present in the stroma act as sentinels, maintaining an $MHCII^{low}$ $CD80^{low}$ $CD86^{low}$ immature phenotype in the centre of corneas *vs* at the periphery where immature and mature DCs coexist[53,54]. Following inflammation, infection or corneal trauma, resident B220+ $CD11c^{low}$ pDCs, $CD34^+$ MHCII myeloid precursors and $CD11b^+CD11c^-$ macrophages, as well as infiltrating DCs recruited from the bone marrow permeate the corneal collagen matrix as part of the protective response[55-57].

In the spleen, the largest secondary lymphoid organ, several resident DC populations including lymphoid DCs, myeloid DCs and pDCs have been identified in both humans[58] and mice[59]. However, relatively little is understood about the complex interactions between the matrix components and DCs occupying different splenic zones that are responsible for the crucial task of maintaining tolerance to self-components. Interestingly, splenic stroma has been shown capable of supporting hematopoiesis of dendritic-like cells from splenic or bone marrow precursors[60]. Within the bone marrow, specialized tissue microenvironments or niches crucial for homeostasis of resident hematopoietic stem cell DC progenitors have been described at vascular sites mediated by associations with endothelial cells or at osteoblast sites[61]. Furthermore, while cellular mechanisms responsible for DC tolerance in certain tissues have been elucidated, the specific nature of the ECM ligands and counterparts that form an important part of this "homeostasis handshake" remain poorly characterized.

Markedly, DCs are absent or at least their presence is debated in certain tissues such as the brain[62], while others have contradicted this finding[63]. It is important to mention that isolations of brain DCs have been contaminated with DCs from the meninges which are themselves very rich in DCs[64], analogous to the retina which has few, if any DCs as opposed to the uvea which is abundantly populated with DCs. Under steady-state conditions normal brain parenchyma was found to have resident $CD11c^+$ $MHCII_{neg}$ ramified cells, possibly differentiated from microglia[63]. Also, brain resident DCs could be removed and differentiated into immature DCs with GM-CSF and to mature DCs with CD40 ligation as shown in[65]. It is important to elucidate how DCs maintain homeostasis and also why they are absent in bone, cartilage and other tissues, yet these tissues are flooded with DCs during inflammation. What are the cues from the ECM that keeps DCs away during homeostasis? How does the ECM contribute towards this diversion/chemorepulsion event? Or do DCs gain entry but then undergo apoptosis? Finally, how is this phenomenon relevant to enhancing the immunocompatibility of artificial stroma utilized in regenerative

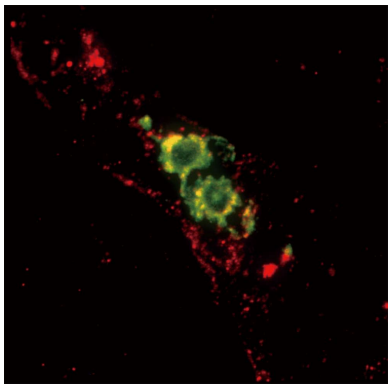


Figure 2 Mature skin dendritic cells egress *via* lymphatics that display secondary lymphoid tissue chemokine (SLC/CCL21) constitutively. Shown here are two mature DC expressing MHC class Ⅱ (green) located within a lymphatic vessel that presents SLC (red). Reprinted from Jakob T, Ring J, Udey MC. Multistep navigation of Langerhans/dendritic cells in and out of the skin. *J Allergy Clin Immunol* 2001; 108: 688-696 Copyright (2001), with permission from Elsevier. Courtesy of Saeki H and Hwang S, National Cancer Institute, Bethesda, MD.

medicine?

IT WORKS BOTH WAYS: DC MODULATION OF MATRICES

Communication between DCs and the ECM is bi-directional. The DCs signal out to the ECM and actively modulate the matrix which affects their ability to migrate, adhere, traffic into lymphatics, or cross the vascular endothelium. Specifically, epidermal LCs migrate towards skin-draining LNs after antigen encounter, a complex transmigration event mediated by C-C chemokine receptor type 7 (CCR7), selectins, integrins and MMP activity to cleave ECM components (Figure 2)[6,40,41]. In the LNs, LCs present antigens in the context of self/non-self to T cells to drive tolerance or immunity, as seen in T_H2-driven atopic dermatitis[66,67]. Markedly, in heparanase deficient mice that are incapable of cleaving heparan sulfate in the ECM and on cell surfaces, the loss of heparanase results in critical defects in facilitating DC migration from skin to lymphatics. Intriguingly, immature DCs in these mice transition to mature DCs and appear more activated. Increased DC activation is possibly due to the compensatory elevation of CCR7 expression and CCR7-CCL19-driven DC activation[68]. Furthermore, DCs co-cultured with fibroblasts in the presence of TNF-α/IL-1β enhanced MMP-9 expression on DCs, important for DC migration *via* degradation of collagen type Ⅳ in basement membranes[6], underscoring the important role played by the ECM in maintaining DC homeostasis and controlling DC localization within tissue. Tissue migratory cDCs display heterogeneity depending on their microanatomical location. For instance skin epidermal migratory LCs are distinct from dermal DC subsets (Langerin+CD11b^{low}, Langerin-CD11b^{-}, and Langerin-CD11b^{low}) and display differential migratory characteristics depending on the nature of the antigen[69]. Following skin infection with HSV, epidermal LCs migrated swiftly away from the epidermis, likely towards skin draining LNs in contrast to dermal DCs which collected in the dermis together with monocyte-derived DCs[70]. On the other hand, skin painting with contact sensitizing substances resulted in differential migration kinetics within different dermal DC subsets and as compared to LCs, suggesting that dermal DCs arrive earlier in cutaneous LNs and are involved in the early response to skin immunization[71]. This observation is supported by another study that demonstrated a vital role for tissue migratory dermal DCs rather than migratory LCs in response to contact hypersensitivity induced by 2,4-dinitro-1-fluorobenzene in terms of their abilities to elicit antigen-specific T cell proliferation. Taken together, these studies highlight the exquisite complexity that tissue migratory DCs demonstrate in their direction of response, migration kinetics and role depending on the specific properties of the matrix that they occupy. In other words, the DC subset that migrate initially to the site of an insult and therefore help shape the overall adaptive response may be profoundly impacted by the anatomical and microenvironmental location of the injury and the resident DC populations present there.

MAJOR ECM COMPONENTS IMPINGING ON DC

Individual constituents of the ECM have been associated with differential impacts on inflammation and immunity. Distinct contributions of the various classes of ECM macromolecules have been reviewed in this section with special emphasis on their unique interactions with DCs to highlight how the specific biology, site in the body, phase of response, form (soluble or particulate), fragment length, post-translational adaptations and enzymatic modifications may vary in healthy *vs* pathological conditions (Table 1). While by no means a comprehensive list, we highlight that ECM components are crucial factors and play distinct roles in directing innate/adaptive responses, focusing on DC/cellular/humoral-orchestrated downstream homeostasis or inflammatory consequences.

Collagen

Collagens represent a major component of the ECM and connective tissue with characteristic Gly-Pro-X repeats, providing support and tensile strength[72]. Collagen type Ⅰ (skin, tendon, bone, interstitial tissues, ligaments, cornea), type Ⅱ (cartilage, vitreous humour) and type Ⅲ (skin, muscle, blood vessels) account for the majority of collagens present in the body[72]. While collagen types Ⅰ, Ⅱ and Ⅲ are present as covalently crosslinked fibrils, notably, type Ⅳ collagen forms a two dimensional reticulum (basal laminaes)[73]. The type and form (soluble or particulate) of collagen appear to be important determinants of their abilities to stimulate DC activation. Soluble collagen types Ⅰ, Ⅱ, Ⅲ coated onto

Table 1 Dendritic cells and the maintenance of homeostasis in different tissues

Tissue	Location	Resident DC in naïve tissue	Resident associated cells/ naïve tissue	Stromal/cellular interactions in immune homeostasis	Ref.
Skin	Epidermis	Cd1a$^+$ Langerin+ Langerhans cells expressing Birbeck granules	Keratinocytes	E-cadherin junctions with keratinocytes, TGF-β production, tolerogenic T_{reg} responses	[6, 40-42]
	Dermis	CD1c$^+$ DC-SIGN+ DEC205$^+$ dermal DC subsets (Langerin+ CD11b^{low}, Langerin-CD11b-, and Langerin-CD11b^{low})	To be elucidated (presumed dermal matrix, fibroblasts)	Pluripotent dermal DC may present antigen, migrate or reside in tissue depending on local interactions	[69, 144]
Intestinal mucosa	LP, Peyer's patches, GALT, SILT	CD103$^+$CD11b$^+$ or CD103$^+$ CD11b- migratory DC, CD103$^+$Sirpα- DC, pDC, CX3CR1$^+$ DC	Macrophages, B cells	Maintain immune homeostasis, induce T_{reg} differentiation, oral tolerance (TGF-β, T_{reg}, T_H2 factors) Gut: Retinoic acid, T_H17 cells LP: indoleamine 2,3-dioxygenase CD83 on DC regulates mucosal tolerance	[5, 48-50, 145, 146]
Liver	Portal tracts, interstitial DC	CD103$^+$DC, CD103$^-$DC, CD103$^-$ CD11b$^+$DC, CD141$^+$ DC (high in healthy liver)	Hepatic stellate cells, sinusoidal endothelial cells, Kupffer cells, hepatocytes	Inhibit DC activation (indoleamine-2,3-dioxygenase expression), repress T cell activation (IL-10, TGF-β) *via* CTLA-4, PD-1	[4, 51, 52,147,148]
Cornea	Central/peripheral corneal stroma	CD11b$^+$ CD11c$^+$ DC, B220$^+$ CD11c^{lo} pDC, CD34$^+$ MHCII myeloid precursors	Stromal Collagen Ⅰ, CD11b+ CD11c- macrophages, keratocytes	Maintain MHCII^{low} CD80^{low} CD86^{low} phenotype under normal conditions	[54-57]
Spleen	Marginal zones	Lymphoid, myeloid and pDC	Macrophages, T cells, B cells (zone dependant)	To be elucidated	[58-60]
Bone marrow	Osteoblastic or vascular niches	Resident hematopoietic stem cell DC progenitors	Osteoblasts, stromal cells and sinusoidal endothelial cells	-	[61]
Retina	Peripheral margins and juxtapapillary areas	Presence of DCs is debated. Few MHCII$^+$ 33D1$^+$ DC observed in naïve brain	Likely migrated in from choroid, ciliary body and meninges	Perivasulcar - around retinal venules (initial site of immune disruption), but not arterioles.	[149]
Brain	Regions of synaptic plasticity and neurogenesis	Presence of DCs is debated. Brain-derived CD11c$^+$ DC	-	-	[62,63,150]
Bone/ cartilage/ vitreous	Not detected	-	-	-	-

DC: Dendritic cell; TGF-β: Transforming growth factor-β; LP: Lamina propria; GALT: Gut-associated lymphoid tissues; SILT: Solitary intestinal lymphoid tissue; MHCII: Major histocompatibility complex class Ⅱ; IL: Interleukin-4.

dishes activated murine/human BMDCs and resulted in elevated costimulatory receptor expression, pro-inflammatory cytokine secretion and allostimulatory capacities[74-76], demonstrating that ECM components can trigger DC activation locally. In contrast, extracted dermal hydrogels composed of basement membrane constituents such as particulate collagen type Ⅳ, collagen type Ⅶ and laminin β3 improved dermal wound healing in a rodent model and mitigated granulation tissue thickness by assisting with wound contraction[77]. Furthermore, in a study comparing the effects of individual matrix components on DC maturation, DCs cultured on plates coated with ECM components fibronectin, collagen, gelatin, or on poly-lysine or polystyrene surfaces were observed to upregulate CD80, MHCII in the presence of pro-inflammatory factors. Interestingly however, on Matrigel (collagen type Ⅳ, laminin, entactin, heparan sulfate proteoglycans)-coated surfaces, the ECM components were able to inhibit DC maturation even in the presence of activating factors[78], suggesting that gelatinous Matrigel derived from murine tumour stroma and mimicking basement membranes promotes DC tolerance to maintain homeostasis under normal conditions.

Glycoproteins - fibronectin, vitronectin, laminin and fibrillin

Glycoprotein constituents of ECM play well defined roles during inflammation. In injury, fibronectin draws cells towards repopulating the wound by exploiting cell surface integrins, while laminin helps in the formation of blood vessels[72]. Interestingly, fibronectin and laminin have been implicated in inhibiting DC maturation. Specifically, human monocyte-derived DCs cultured in the presence of pre-adsorbed fibronectin and laminin retained a less mature phenotype with enhanced endocytic capacities (Figure 3)[79]. On the other hand, modified presentation of Arg-Gly-Asp (RGD) integrin-binding sequence of the ECM glycoprotein fibrillin in microfibrils disrupted murine pDC adherence and increased its activation (IFN-α, IL-6), plasma cell and B cell accretion and autoantibody secretion, skewing of

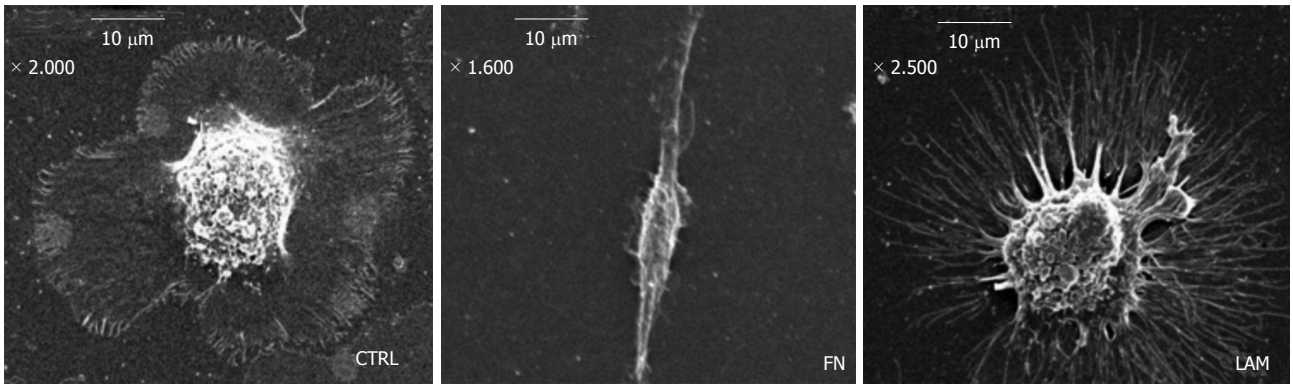


Figure 3 Scanning electron microscope images demonstrate that dendritic cells cultured on fibronectin or laminin for 48 h exhibit widely differential morphologies. Taken in part from García-Nieto S, Johal RK, Shakesheff KM, Emara M, Royer PJ, Chau DY, Shakib F, Ghaemmaghami AM. Laminin and fibronectin treatment leads to generation of dendritic cells with superior endocytic capacity. *PLoS One* 2010; 5: e10123. Copyright (2011), published under the Creative Commons Attribution (CC BY) license. FN: Fibronectin; LAM: Laminin.

T_H subsets and ultimately enhanced dermal fibrosis, showing a role for fibrillin in instigating pro-inflammatory, pro-fibrotic programmes[80].

Non-proteoglycan polysaccharides

HA: HA a copolymer of GlcNAc and GlcUA plays dual roles both as supporting meshwork of lymphatics as well as that of a potent danger signal, based on the fragment length[81]. Breakdown of long glycosaminoglycan HA resulting in the formation of small HA fragments activated skin DCs. Higher serum and lymphatic HA levels have been associated with reduced DC maturation and feeble tumour responses correlating to higher HA in tumour ECM[82]. Hyaluronic acid is a significant non-immunogenic component of healthy ECM linked to wound healing, inhibition of inflammation and angiogenesis. Hyaluronic acid hydrogels enhanced healing after myocardial infarction in rats by decreasing collagen production and increasing vascular endothelial growth factor levels[83].

Modulators: Periostin plays an anti-inflammatory role in IgE-mediated airway hyperresponsiveness and allergy *via* upregulating active TGF-β and therefore inducing differentiation of T_{regs}[84]. Tenascin C is an ECM glycoprotein not normally detectable in healthy adult tissues but is present in pathological conditions such as arthritis[85] and myocarditis[86]. In tumours, tenascin C has been implicated in epithelial mesenchymal transition and migration of cancer cells[87]. Secreted protein acidic and rich in cysteine is a Ca^{2+} binding matrix glycoprotein involved in organization of germinal centres of LNs and essential for follicular DCs to receive necessary cues to induce T_H17 differentiation as shown in a model of experimental autoimmune encephalomyelitis[88]. Thrombospondin 1-DC axis is a negative regulator of inflammation associated with elevated levels of anti-inflammatory mediators (PGE-2, TGF-β). It is critical towards maintaining homeostasis and serves to resolve inflammation during wound healing[89].

MMPs

In injury, enzymes are involved in matrix turnover and remodelling, needed for cell entry and egress and proliferation, vasculogenesis and angiogenesis[72]. Tumour enlargement and dissemination involve interplay between tumours, immune cells and ECM. Active MMP-2 acts as an endogenous anti-inflammatory mediator as evidenced by anti-inflammatory T_H2 profile of MMP-2-expressing $CD4^+$ T cells that infiltrate tumours and the roles of MMP-expressing DCs in inducing this profile *via* OX40L and inhibition of IL-12p70 production[90,91].

Post-translational modifications of ECM components and effects on DC homeostasis

Post-translational modifications including glycation, carbamylation and citrullination have implication in diabetes, kidney fibrosis and inflammatory conditions such as rheumatoid arthritis respectively[92], *via* interactions with DC C- type lectin receptors (CLRs), a class of PRRs[2]. Alterations of the ECM are strongly linked to altered ligand binding and cellular interactions[93]. Inhibition of terminal fucosylation alters macrophage phenotype from pro- to anti-inflammatory, demonstrating how immune homeostasis can be compromised by altered glycosylations of ECM components[94]. Altered fucosylation and exposure of glycans normally "buried" on serum IgG have been implicated in systemic lupus erythematosus progression[95], while changes in sialylations may transform it from being pro- to anti-inflammatory[96]. Interestingly, mucosal surfaces of the human female reproductive tract display glycation patterns analogous to those seen on metastatic cells or on efficacious pathogens in order to promote anti-inflammatory responses for survival of placenta and human sperm[97], reinforcing the observations that host proteins can be altered to present tolerizing or activating glycosylation patterns as reviewed in[98].

DC RECEPTORS FOR ECM

DCs express many receptors which interact with tissue

or matrix components during homeostasis as well as with their breakdown products[78], some of which have been reviewed here and summarized in Table 2. Immature DCs express adhesion complexes to bind different structural ECM components: CD49a/CD29 and CD49b/CD29 to collagen and laminin; CD49c/CD29 to collagen, laminin, fibronectin and thrombospondin; CD49d/CD29 and CD49e/CD29 to fibronectin; CD49f/CD29 to laminin; CD41, CD51 and CD61 to fibrinogen, fibronectin, vitronectin and thrombospondin[78]. Besides expressing adhesion molecules to direct their migration and localization within tissues, DCs express extracellular and cytoplasmic PRRs such as TLRs, RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) that respond to pathogens[99,100]. Major PRRs such as TLRs bind bacterial and viral nucleic acids, lipopetides, and lipopolysaccharide and initiate signalling cascades triggering DC maturation[101]. Cytoplasmic RLRs recognize bacterial and viral nucleic acids while NLRs such as NOD1 and NOD2 bind bacterial peptidoglycans[102]. Endocytic scavenger receptors recognize modified and unmodified lipoproteins[103].

Another class of PRRs, endocytic CLRs bind glycosylated moieties in a Ca^{2+} dependant manner, and internalize and present both self and non-self on MHC molecules[104,105]. CLRs recognise glycations on ECM constituents - N-linked glycans present on glycoproteins as well as O-linked glycans on collagens[105]. Importantly, antigen uptake by CLRs resulting in TLR ligation results in the generation of antigen-specific immunity, while in contrast, CLR-mediated recognition alone facilitates homeostasis and tolerance to tissue antigens, as discussed in this review[105]. There is growing evidence that CLRs play significant roles in regulating immune tolerance in the gut[106]. Dectin-1 and galectin-3 maintain tolerance to gut mucus by repression of NF-κB[107]. Tolerogenic DCs recognize GalNAc on tumours by MGL (macrophage galactose/N-acetylgalactosamine-specific C-type lectin) binding[105] and mannose receptor expressed by DCs has been implicated in maintaining immune homeostasis[108]. Furthermore, $MGL1^+$ and $MGL2^+$ cells were detected in various tissues under normal conditions suggesting that they play an active role in tightly controlling DC homeostasis in tissues regularly exposed to antigens including thymus, intestine, stomach, trachea and skin[109]. Modified glycosylation patterns alter CLR binding and contribute towards immune evasion by impacting CLR-TLR cross talk[105].

"Homeostatic danger signals" were defined in a recent review as disruptions in tissue steady-state that stimulate DC activation, typically occurring during inflammation[110]. Endogenous DAMPs released or activated during injury or surgical trauma include degraded ECM constituents such as fibronectin, fibrinogen, HA[111,112] heparan sulfate[113], biglycan[114,115], versican[116] activate DCs mediated by TLRs resulting in pro-inflammatory outcomes[117,118]. Heparan sulfate, TLR4 agonist stimulates DC activation and enhanced allostimulation *in vitro*[113]. Blocking heparan sulfate serum levels with alpha-1-antitrypsin reduced extent of graft *vs* host diseases in mice[119]. Biglycan is released from ECM during tissue damage or may be produced by inflammatory cells and activates DCs *via* TLR2/4[115], MyD88, TRIF as shown in myocardial matrix[120]. Chondroitin sulfate proteoglycans regulate immunity in the CNS[121]. Breakdown of long glycosaminoglycan HA resulting in the formation of small HA fragments activated skin DCs in a TLR4-mediated manner[122]. Another ECM component, tenascin C initiates TLR4-mediated DC activation and generation of T_H17 cells[85,86].

DC receptors that recognize collagen, a major constituent of the ECM, may be activating (discoidin domain receptors[74,75], mannose family receptors, glycoprotein Ⅵ) or tolerogenic [CD305/leukocyte-associated Ig-like receptor 1 (LAIR-1)[123]]. LAIR-1 binds soluble adsorbed collagen (hydroxyproline in Gly-Pro-Hyp) and interferes with DC differentiation in an immunoreceptor tyrosine-based inhibitory motifs -mediated manner[124]. LAIR-1 may play an important role in maintaining homeostasis and has been shown to be upregulated on tumour-associated DCs[125]. On the other hand, soluble adsorbed collagen types Ⅰ, Ⅱ, Ⅲ, ligands of osteoclast-associated receptor were shown to activate human monocyte-derived DCs and triggered upregulation of maturation markers, TNF and TLR signalling demonstrating that ECM components can trigger DC activation locally, important in the context of DC differentiation into bone-degrading osteoclasts in the synovial tissues of rheumatoid arthritis patients[76].

INTELLIGENT BIOMATERIAL DESIGN TO MIMIC ECM IN TISSUE REGENERATION

In recent years, tissue engineering strategies have been proposed to address the shortfall of utilizable donor tissues for transplantation. The main objective is to generate functional, viable tissue substitutes that are well-integrated long-term in a site-specific manner. Several regenerative medicine approaches are ECM-based and some include the use of processed whole tissues such as decellularized stroma or human amniotic membrane where the intrinsic mechanical and functional properties of the matrix can be exploited to promote tissue regrowth. Other strategies employ ECM-derived biopolymers from mammalian and other sources including collagen, fibrin, chitin and chitosan, taking advantage of the dynamic, flexible nature of these scaffolds in directing cellular engineering of skin, cartilage, bone and nerve[126]. As a next step, bio-interactive implants comprising polymers coated with ECM proteins such as laminin, fibronectin, collagen or with grafted or tethered cell adhesive peptides have been proposed[127].

Overcoming the host immune/inflammatory response remains a significant challenge to the long-term success of ECM-based implants. Most of this work

Table 2 Dendritic cell interactions with extracellular matrix components

Class of component	ECM component	DC responses	DC receptors	Overall impact
Collagen	Soluble collagen Ⅰ [74]	Murine BMDC upregulated CD86, IL-12, antigen uptake	DDR2	Pro-inflammatory
	Soluble collagen Ⅰ [75]	Human MDDC increased IL-12p40, TNF-α, IFN-γ	DDR2	Pro-inflammatory
	Adsorbed collagen Ⅰ, Ⅱ, Ⅲ[76]	Human MDDC increased maturation markers, pro-inflammatory cytokines, allostimulation	OSCAR	Pro-inflammatory
	Dermal hydrogel (laminin β3, collagen Ⅳ, Ⅶ)[77]	Decreased width of granulation tissue	-	Skin regeneration, anti-infl.
	Adsorbed fibronectin, collagen Ⅰ, gelatin, Matrigel[78]	Murine myeloid DC on Matrigel were less mature (maturation marker, cytokines, morphology)	Adhesion complexes (CD29, CD49a-f, CD41, CD51, CD61)	Differential effects – Matrigel less inflammatory *vs* collagen Ⅰ
	Collagen-like motifs in complement C1q[124]	Inhibits MDDC differentiation, TLR activity of pDC	LAIR-1	Anti-inflammatory
Glycoproteins	Pre-adsorbed laminin, fibronectin[79]	Human MDDC remained immature (maturation marker, high endocytosis)	Mannose receptor, DC-SIGN	Anti-inflammatory
	Modified Arg-Gly-Asp (RGD) on fibrillin[80]	Murine pDC adherence, TGB-β secretion increased in systemic sclerosis model	Integrins	Pro-fibrotic
Proteoglycans	Heparan sulfate[113,119]	DC maturation increased (morphology, costimulatory factors, T cell stimulation)	TLR4	Pro-inflammatory
		In GVHD blocking HS with alpha-1-antitrypsin limited alloreactive T cells	-	Pro-inflammatory
	Chondroitin sulfate[121]	Impact immunity in CNS pathologies	-	Pro- and anti-inflammatory
	DAMPs[111-113,117,118]	Activate DC	TLRs	Pro-infl.
Non-proteoglycan polysaccharides	Hyaluronan[82]	Increased hylauronan corresponds to decreased murine DC activation	-	Anti-inflammatory (tumours)
	Natural polymer hyaluronic acid[129,130]	Decreased DC maturation (maturation markers, cytokines, allostimulation)	-	Anti-inflammatory
Modulators	Secreted protein acidic and rich in cysteine[88]	Organization of germinal centres in LNs for T_H17 by follicular DC	-	-
	Thrombospondin-1[89]	DC-derived thrombospondin inhibits resolution of inflammation	CD47, CD36	Anti-inflammatory
Enzymes	Matrix metalloproteinases[90]	Endogenous MMP-2 prime DC to T_H2 (IL-12p70)	-	T_H2 profile
	Tissue transglutaminases[151]	Influence DC activation (concentration-dependant)	-	Pro- and anti-inflammatory
Glycosylation modifications	Gut mucous[107]	Decrease in DC activation by inhibition of NF-κB	Dectin-1, galectin-3	Anti-inflammatory
	Tissue matrix in skin thymus, trachea[109]	Steady state homeostasis	$MGL1^+$ $MGL2^+$	Anti-inflammatory

DC: Dendritic cell; ECM: Extracellular matrix; OSCAR: Osteoclast-associated receptor; DDR: Discoidin domain receptors; IL: Interleukin-4; TNF-α: Tumour necrosis factor-α; IFN-γ: Interferon-γ; LAIR-1: Leukocyte-associated Ig-like receptor 1; TLR: Toll-like receptor; MGL: Macrophage galactose/N-acetylgalactosamine-specific C-type lectin.

addresses the effect of various biomaterials on the inflammatory response and particularly macrophage behaviour and its effects on the ECM. However, little work has been done on how biomaterials affect DC behaviour and function. Information is needed in this area since biomaterials may not only act as allo and xenoantigens but can directly behave like DAMPS (see Introduction) and thereby promote autoimmune responses through host tissue damage. Since tissue *via* the ECM tightly regulate DC homeostasis and inflammation, this directly impinges on how artificial matrices affect DC behaviour and hence the balance between immunogenicity and tolerance. Artificial stroma and their components may activate or suppress DCs, induce DC differentiation, promote or inhibit fibrosis or change DC interactions with other cells, *e.g.*, other inflammatory cells or activate the adaptive immune response *e.g.* as an "autoimmune" response when human altered matrices are implanted in humans (or mouse into mouse, *etc.*). Studies have compared the individual effects of natural polymers on DC responses. Human monocytes differentiated to DCs in the presence of the natural, biocompatible polymer chitosan, a polysaccharide derived from the exoskeleton of crustaceans or cell walls of fungi, were activated to a pro-inflammatory state (higher CD86, TNF-α, IL-

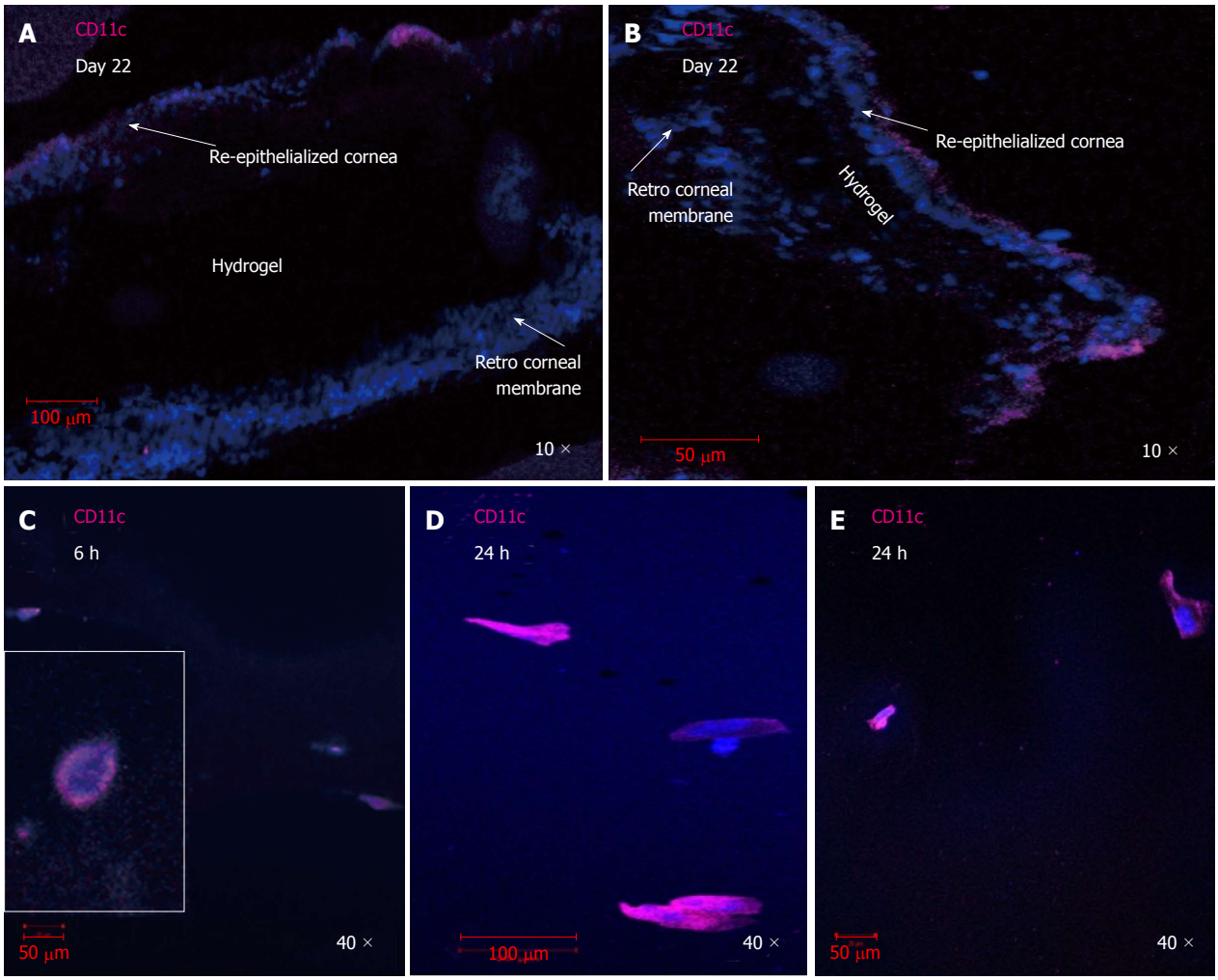
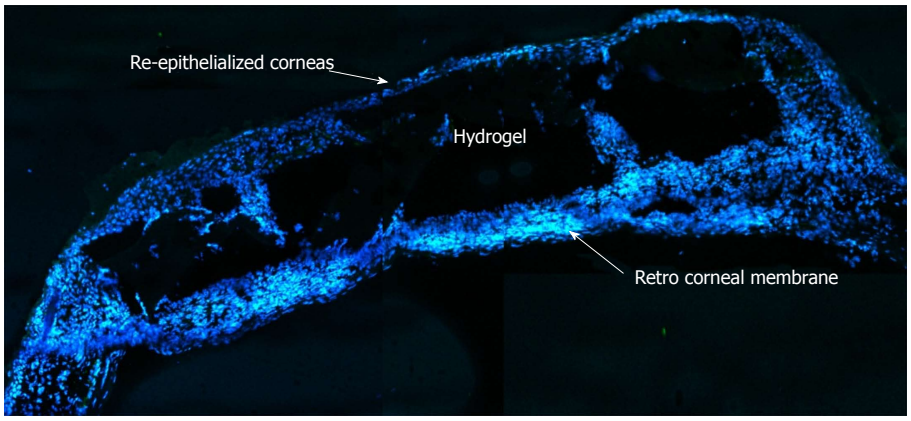


Figure 4 CD11c$^+$ dendritic cells are involved in the host response to transplanted hydrogels. CD11c$^+$ DC were detected in re-epithelialized layers surrounding RHCIII hydrogels transplanted in murine corneas, 22 d post transplantation (A, B). CD11c$^+$ dendritic cells infiltrated hydrogels transplanted into murine corneas as early as 6 h (C) or 24 h (D, E) after transplantation and showed a transformation in morphology from rounded immature DC to well-differentiated DC at the latter time point after interacting with the three dimensional crosslinked collagen matrix. Images were taken within the hydrogel in (C-E) as shown in the schematic. DAPI: Nuclear staining shown in blue; CD11c in magenta; DC: Dendritic cell.

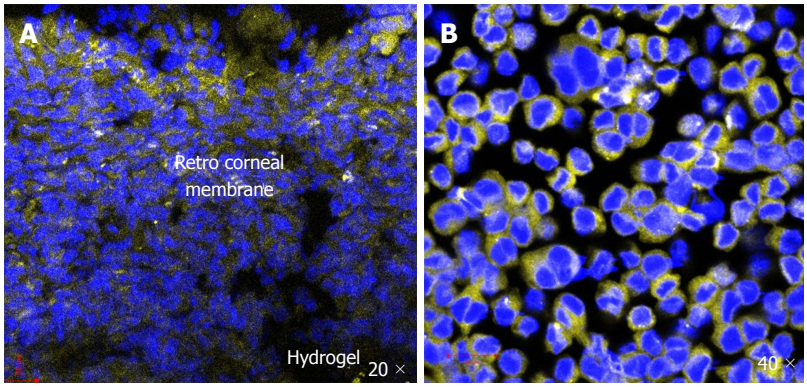
1β and lower IL-10 levels)[128]. Also, it was observed that while alginate and hyaluronic acid were less maturing to DCs, the opposite effect was observed with chitosan or agarose[129,130], implying that specific ECM mimetics can have applications in vaccine delivery or in tissue engineering whether host immune responses are desired or not, as suggested in[129]. Surprisingly, regenerative medicine approaches to reconstruct heart valves using xenogeneic porcine or bovine collagen and elastin, did not induce human DC maturation (low CD83 expression and TNF-α secretion)[131]. Hydrogels fabricated from lyophilized constituents of porcine dermal ECM were coated onto polypropylene meshes as a means of reducing the inflammatory responses associated with these non-biodegradable materials. The presence of ECM hydrogels facilitated decreased recruitment of CD86$^+$ CD68$^+$ M1 macrophages by day 14 post implantation in rats and decreased collagen type Ⅰ deposition related to wound healing responses[132]. HA was electrospun into nanofibers to assist the adherence and survival of NIH-3T3 fibroblasts to mimic ECM properties to support cell adhesion[133].

ECM-based scaffolds have been developed to boost tissue repair and reconstruction. Since collagen is a major ECM component in most tissues, different strategies have been employed to generate three dimensional fibrillary collagen matrices, including plastic compression of hydrated polymerized collagen and fluid expulsion, using contractile properties of activated fibroblasts, as discussed in[134]. Regenerative biomaterial scaffolds composed of clinical grade recombinant human collagen hydrogels crosslinked with water soluble carbodiimides have been fabricated to mimic the type Ⅰ and Ⅲ collagens predominantly present in natural corneas that are crosslinked with glycosaminoglycans[135]. These ECM mimetics are cell free, chemically well characterized, resistant to biodegradation and mimic natural corneas in terms of optical and mechanical properties of corneas. They retained optical clarity in partial[136] or full-thickness[137] corneal transplants in animal models



Day 22 post transplantation
aSMA, DAPI, 10 ×

Figure 5 Merged images showing the formation of dense retro-corneal membrane separating corneal hydrogel from lens and posterior areas of eye, shown on day 22 after transplantation of murine corneas with RHCIII hydrogels. Newly generated membrane exhibits presence of alpha smooth muscle actin, likely produced by myofibroblasts recruited during the wound healing process. Tissue ingrowths into the RHCIII hydrogel are evidence of active remodelling of the artificial matrix scaffold by immune/inflammatory cells and ECM components, towards long-term integration with natural tissue. DAPI: Nuclear staining shown in blue; Alpha smooth muscle actin in green; ECM: Extracellular matrix.



Day 22 post transplantation
Tenascin C, DAPI

Figure 6 Murine corneas transplanted with RHCIII hydrogels stained positive for extracellular matrix constituent tenascin C in the retro-corneal membrane, a marker of epithelial to mesenchymal transition, indicative of active wound healing (A) and WEHI-164 murine fibrosarcoma cell line cultured with 5 ng/mL transforming growth factor-β1 for 48 h also produced tenascin C (positive control) (B). DAPI: Nuclear staining shown in blue; Tenascin C in yellow.

and promoted corneal cellular and nerve regrowth. ECM scaffolds have also been fabricated to elucidate mechanisms underlying cell-matrix interactions in physiologically relevant settings. DCs were recruited to murine corneas transplanted with RHCIII hydrogels and could be detected surrounding and within the artificial matrix, demonstrating their involvement in the host response (Figure 4)[138]. Also, RHCIII hydrogels implanted in murine corneas underwent remodelling by cellular and ECM components as part of the wound healing process (Figures 5 and 6)[138]. A three dimensional model composed of epithelial cells, fibroblasts generating ECM components such as tropoelastin, vimentin, collagen type Ⅳ and laminin and DCs was developed to recapture the complexity and architecture of DC interplay with lung tissue mucosa towards maintaining homeostasis[139]. Lung epithelial cells are no longer considered mere physical barricades against foreign allergens but key players in mediating DC responses and T_H2 responses as reviewed in this paper[140].

Three dimensional ECM mimetics have shown promise in the transition from bench to bedside. Notably, RHCIII scaffolds were employed as partial thickness corneal transplants in a 4-year clinical study in 10 patients and demonstrated minimal rejection, enhanced stability, epithelial, stromal cell and nerve regeneration (human allografts) (Figure 7)[141]. Remarkably, DCs were not recruited into transplanted RHCIII hydrogels but in contrast were present in donor human allografts[141]. In another study, bone substitute P-15 comprising bone mineral calcium phosphate and cell-interactive peptide of collagen type Ⅰ acted as a promising alternative to allografts in its ability to repair non-union fractures as exhibited in a pilot clinical study with 22 patients, an example of an ECM mimetic that has successfully reached the bedside[142]. A randomized clinical trial with 120 patients showed that natural tissues derived from

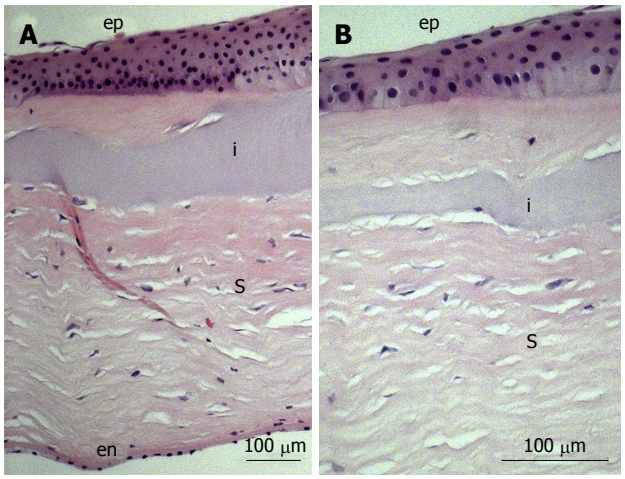


Figure 7 Immunohistochemistry image of biointeractive RHCIII hydrogel in a lamellar keratoplasty and removed after 4 years, upon regrafting of the patient. Notably, regrowth of characteristically stratified corneal epithelium (ep), layered stroma (s) and endothelial monolayer (en) left intact during transplantation, can be observed (A). A portion of the RHCIII implant (i) is visible in a higher magnification image (B), displaying a uniform assimilation with the native stroma in a dynamic, ongoing remodelling process. Reprinted from Fagerholm P, Lagali NS, Ong JA, Merrett K, Jackson WB, Polarek JW, Suuronen EJ, Liu Y, Brunette I, Griffith M. Stable corneal regeneration four years after implantation of a cell-free recombinant human collagen scaffold. *Biomaterials* 2014; 35: 2420-2427. Copyright (2014), with permission from Elsevier.

porcine small intestinal mucosa consisting mainly of collagen along with other macromolecules, active forms of basic fibroblast growth factor and TGF-β, enhanced healing[143]. While promising strides have been made, several challenges remain including gaining successful integration of scaffolds into host, retaining long-term stability and functionality and obtaining immune acceptance. Exploiting our knowledge of DC-ECM interactions would be an important way forward.

CONCLUSION

We have reviewed the body of evidence describing interactions between DCs and the ECM and the constantly changing role of the latter in directing DC responses in normal conditions *vs* in inflammation. These mechanisms may be active or reactive. While they offer us a glimpse of the numerous ways that the ECM restrains DCs to play very precise, context-dependant roles, there are probably many more aspects as yet undiscovered. It is possible that the decisions made by individual tissues in allowing DC to enter and reside in them or not and how and why this changes when the tissue is under attack will offer important insights into optimal design of artificial stroma.

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REVIEW

Macrophage populations and self-renewal: Changing the paradigm

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Abstract

The origin of macrophages has been considered since several decades to be a continuum from bone marrow (BM) to tissue *via* monocytes as precursors. The development of new tools such as genetic lineage tracing, parabiosis and BM chimeras changed the paradigm of macrophage origin. In steady state, most resident macrophages are of embryonic origin, whereas a monocyte origin remains prominent in pathological conditions. The findings of a proliferation of mature macrophages will oblige us to reappraise the relationship between proliferation and differentiation in macrophages. This review is based on the recent explosion of high impact articles on macrophage biology. It summarizes new data on the origin of macrophages and their self-renewal potential in steady states. While monocytes are required for intestinal macrophage development, the microglia is independent of monocyte influx and skin macrophages provide an excellent model of the balance between monocyte input and self-renewal. In addition, macrophage proliferation requires intrinsic and extrinsic factors including growth factors and cytokines. It also analyzes the impact of this new paradigm in human diseases such as athrosclerosis, cancer, infectious diseases and neurodegenerative diseases. In atherosclerosis, the finding of macrophage proliferation within the lesions will change our understanding of disease pathophysiology, this new paradigm may have therapeutical impact in the future.

Key words: Macrophages; Self-renewal; Proliferation; Homeostasis; Diseases

Core tip: The emergence of revolutionary technologies in myeloid cell research has deeply changed the paradigm of macrophage activation. It was believed that macrophage derive from myeloid precursors *via* circulating monocytes. Now, we can propose that resident macrophages are of embryonic origin in steady state whereas monocytes are recruited in pathological conditions. The second strong idea was that mature macrophages are unable to proliferate; we have strong evidence that macrophages can proliferate, which is

the basis of self-renewal. The consequences of these new concepts will lead us to reappraise the role of macrophages in pathologies.

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INTRODUCTION

Historical point of view

The initial model of macrophage differentiation was proposed by Ralph Van Furth and Zanvil Cohn[1] in 1968. Tissue macrophages arise from bone marrow (BM) progenitors *via* blood monocytes as intermediates[2]. However, this popular model is probably insufficient to describe how macrophage populations grow and mature.

The introduction of new methods including genetic lineage tracing, parabiosis and BM chimeras as well as BM transplantations in animals and myelo-ablative irradiation or chemotherapy in patients enable a reappraisal of macrophage origin dynamics[3]. We will review the diversity of macrophage populations, the contribution of the self-renewal process to macrophage dynamics and the consequences on our understanding of human pathologies.

Macrophage populations

The mononuclear phagocyte system consists of monocytes, macrophages and dendritic cells (DCs), which exhibit different morphologies, phenotypic characteristics and functions. For several years, it was believed that monocytes were released from BM into the circulation and that they were the precursors of macrophages and DCs[4].

In the past 20 years, use of specific membrane markers has allowed the discrimination of different subpopulations among mononuclear phagocytes. Hence, murine inflammatory monocytes are characterized by the high expression of LY6C, CCR2 and low level of CX_3CR1. In contrast, patrolling monocytes are characterized by the low expression of LY6C and CCR2 and high density of CX_3CR1. A similar heterogeneity has been found in humans; the most prevalent population of so-called classical monocytes expresses high levels of CD14 and is equivalent to $LY6C^{hi}$ in mice.

The level of CD14 and CD16 membrane expression enables the identification of two minor subsets of monocytes, $CD14^{hi}CD16^{lo}$ (also called intermediate monocytes) and $CD14^{lo}CD16^{hi}$ (called non-classical monocytes) respectively, which are the equivalent of $LY6C^{lo}$ monocytes in mice[5]. It has been recently found that the relative proportion of human monocyte subsets is modulated during inflammatory and infectious diseases[6,7], although the role of these different monocyte subsets in these pathologies remains to be elucidated. The situation is even more complex for tissue macrophages. Indeed, macrophages are classified according to the type of tissue: Osteoclasts in bone, alveolar macrophages in lungs, Kupffer cells in liver, intestinal macrophages in the gut, microglia in the nervous system), placenta macrophages and macrophages in secondary lymphoid organs[8,9]. These macrophages exhibit a diversity of functions from host defense to metabolism and tissue remodeling.

Besides these resident macrophages, macrophages can be locally recruited in response to injuries and their phenotypic and functional characteristics will depend on the type of injury. Using some phenotypic features enables the distinction of recruited macrophages from resident cells. The former exhibit a low expression of F4/80, CD64, MerTK, CD14, are mobile and short-living cells; in contrast, the latter highly express F4/80, CD64, MerTK, CD14, are long-living but can be of yolk sac (YS) or hematopoietic stem cell (HSC) origin (see below). The responses of macrophages to different stimuli have led to the concept of macrophage polarization, which allows a classification of functional macrophage subsets. A reductionist model of activation has resulted in a definition of M1 macrophages that reflects the Th1 immune response and M2 macrophages that reflects the Th2 immune response[10]. The lack of specific markers of M1 and M2 macrophages, respectively, has made the identification of these functional subsets in *in vivo* conditions difficult[11]. However, the use of high throughput methods such as microarray permitted the identification of transcriptional signatures that would require functional validation[12]. Studies of networks based on gene expression profiling have generated a resource data set to assess transcriptional regulation during macrophage activation by comparing diverse sets of agonists on a single microarray platform. Network modeling extends the current M1 *vs* M2 polarization model to a spectrum model with at least nine distinct macrophage activation programs[13]. In addition, the InnGen project has enabled the sorting of tissue macrophages from C56BL/6 mice and the analysis of their gene expression program with whole genome microarray. This approach has revealed a considerable diversity among macrophage populations, which is higher than the distance between macrophages and DCs. This diversity is illustrated by the expression of unique transcripts according to each macrophage location. As an example, this bioinformatics approach has revealed that Langerhans cells are close to BM-derived macrophages but surprisingly failed to cluster with macrophages[14]. The introduction of mass cytometry allowed a more precise analysis of murine myeloid cells. Indeed, alveolar macrophages, microglia and red-pulp macrophages are populations distinct from the other macrophages[15]. It is likely that new data will profoundly change our understanding of the relationship between the diversity of macrophage populations and their origin.

Table 1 Approaches to dissect the origins of macrophage lineages

Methods and tools	Results
Membrane markers	CX3CR1^{hi}/F4/80^{hi}/CD11b^{lo}: YS macrophages CX3CR1^{lo}/F4/80^{lo}/CD11b^{hi}: HSC macrophages
Transcription factors	MYB^{+}: HSC macrophages MYB^{-}: YS macrophages
Depletion (clodronate, Abs)	Non-specific depletion with clodronate. The CCR2$^{-/-}$ mice that are depleted from circulating monocytes exhibit normal tissue macrophage populations
Genetic fate mapping techniques: RUNX1	Early expression of RUNX1 in YS derived macrophages and identification of embryonic macrophages in adulthood (microglia, Langerhans cells)
Genetic fate mapping techniques: FLT3	Identification of a HSC stage in differentiation: + for monocytes and - for tissue macrophages
Genetic fate mapping techniques: CSF1R	Labeling of 30% YS derived macrophages in the embryo and similar persistence in adult microglia
Genetic fate mapping techniques: CX3CR1	labeling of monocytes and miocroglia
Parabiosis	Replacement of resident macrophages by chimeric monocytes
Sublethal irradiation and bone marrow transplant	Chimerism in blood monocytes without eradicating resident macrophages. Risk of inflammation and membrane leakage

YS: Yolk sac; HSC: Hematopoietic stem cell. This Table describes the methods to identify the origin of mononuclear phagocytes and the major results. It refers to recent reviews[16-18].

NEW TOOLS TO DETERMINE THE ORIGIN OF MACROPHAGES: MONOCYTES *VS* MACROPHAGES

New tools have emerged these latter years to investigate the origin, the homeostasis and the functions of mononuclear phagocytes[16,17]. We will illustrate these methodological advances with a few examples. The study of mouse embryogenesis enables a chronological dissection of macrophage origin. First, the macrophages appear in YS in which primitive hematopoiesis occurs. Then, fetal liver and BM are populated by HSCs, which represent another source of tissue macrophages[18]. This dynamic and the dual origin of tissue macrophages (YS *vs* HSC) have been reported in a growing number of important papers[18,19]. These results question the role of monocytes in tissue colonization in both homeostasis and situations of danger. Clinical features highlight the new paradigm. Many tissue macrophage populations are not affected in patients with monocytopenia due to leukemia[20] or immune deficiencies[21].

The use of the radioelement ^{89}Sr which targets monopoiesis does not reduce tissue macrophage content in the lung and liver of mice[19]. Similarly, the depletion of circulating monocytes in CCR2$^{-/-}$ mice has a limited impact on tissue macrophage populations[19]. Using genetic fate mapping techniques based on a recombination-induced expression of reporter genes under the control of a constitutive promoter (RUNX1, CSF1R, FLT3) enables identification and tracking of different embryonic macrophage populations into adulthood (Table 1)[18]. Nevertheless, the specificity and the efficiency of these approaches, such the labeling of YS-derived macrophages with RUNX1^{CreER} or with Csf1r^{CreER}, are questionable. Although FLT3-Cre labels specifically blood monocytes, FLT3-Cre negative tissue macrophages are also observed in HSC-derived macrophages[18]. These molecular tools have provided important data and a model was recently proposed in mice: Primitive macrophages would arise from erythro-myeloid progenitors present in YS. These macrophages are the first wave of colonization of the brain and other fetal organs. A second wave would be characterized by the development of fetal monocytes in fetal liver; these latter cells would be the source of resident macrophages with the exception of the brain[22,23]. These major findings remain limited to murine models and their transposition to humans is an important scientific challenge.

MACROHAGE PROLIFERATION

If tissue macrophage renewal does not result from monocyte influx, their proliferation is necessary. The proliferation of transformed lines of macrophages is well established, but their use is limited by the loss of macrophage functions and their poor differentiation compared with mature macrophages. Michael Sieweke's group recently reviewed the self-renewal mechanisms of mature macrophages and identified extrinsic and intrinsic factors[3] (Figure 1). Among the extrinsic factors, the macrophage colony-stimulating factor (M-CSF) occupies a privileged position. The number of tissue macrophages is reduced in animals bearing mutations in M-CSF such as op/op mice and tl/tl rats and in mice deficient for M-CSF receptors (M-CSFR); the efficiency of macrophage depletion is higher in mice deficient for M-CSFR[24]. M-CSFR binds with not only M-CSF but also interleukin (IL)-34. Produced by neurons and keratinocytes, IL-34 is a good candidate for controlling the homeostasis of microglia and Langerhans cells[25] but seems more critical in the homeostasis of Langerhans cells than of microglia[26]. It is likely that the imbalance between M-CSF and IL-34 accounts for the differences in the macrophage replenishment of the skin and nervous system.

Granulocyte macrophage colony-stimulating factor (GM-CSF) is another important cytokine involved in the turnover of tissue macrophages. This has been clearly demonstrated with macrophages derived from

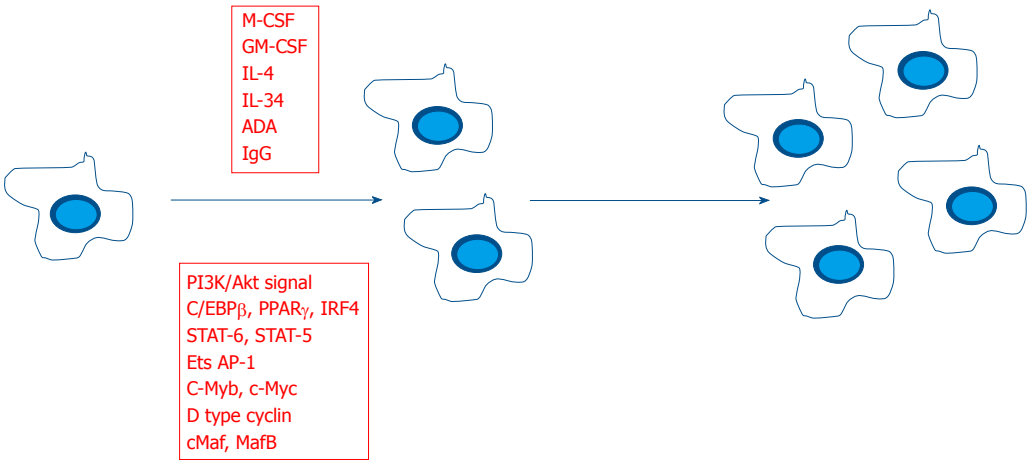

Figure 1 Extrinsic and intrinsic factors involved in macrophage proliferation. M-CSF: Macrophage colony-stimulating factor; GM-CSF: Granulocyte macrophage colony-stimulating factor; IL: Interleukin; PPARγ: Peroxisome proliferator-activated receptor γ.

fetal liver macrophages with self-renewing potential. These cells are obtained by the culture of fetal liver and grow exponentially in the presence of GM-CSF before differentiation. The removal of GM-CSF blocks their proliferation[27]. *In vivo*, the peritoneal administration of GM-CSF also induces the proliferation of peritoneal macrophages[3]. In addition, it is likely that GM-CSF is involved in the control of the alveolar macrophage population[28].

IL-4 shares with M-CSF the ability to polarize macrophages towards a M2 phenotype and is associated with the self-renewal of macrophages. IL-4 is probably sufficient to induce the proliferative expansion of macrophages in serous cavities, the liver, spleen and lungs[29]. The administration of IL-4 in mice causes macrophage proliferation and their accumulation in the liver, spleen and BM[30,31]. In contrast, IL-4 is unable to induce the proliferation of macrophages *in vitro*, suggesting that IL-4 acts in concert with other cytokines. Finally, among the extrinsic factors involved in the self-renewal of mature macrophages, one can evoke adenosine deaminases, known for their role in the regulation of adenosine levels which are associated with monocyte-to-macrophage differentiation and macrophage proliferation[32].

The intrinsic factors playing a role in the self-renewal of macrophages include the signaling pathways of IL-4, IL-34, M-CSF and GM-CSF. In addition, transcription factors such as c-Myb and c-Myc, known for their role in cell proliferation, play a role in monocyte differentiation. Although their ectopic expression in mature macrophages re-initiates the cell cycle[3], they are not involved in the proliferation of mature macrophages[33]. The transcriptional factor Gata6 is specifically expressed by self-renewing peritoneal macrophages but not by monocytes recently recruited into the peritoneum after challenge. Gata6 deficiency impairs peritoneal macrophage renewal during steady state and in response to inflammatory challenge compromising the resolution of inflammation. Gata6 targets genes involved in cell proliferation since their expression is altered in macrophages from Gata6-deficient mice[34]. Other transcription factors regulate macrophage proliferation *via* their cooperation. The cooperation of cMyc and Klf4 and MafB and cMaf seems necessary for macrophage self-renewal as described for stem cells[3]. Hence it has been reported that macrophages isolated from MafB- and cMaf-double deficient mice divide indefinitely; the self-renewal depends on cMyc and Klf4[19]. Taken together, these results suggest that other tissue-specific mechanisms may be identified in the future to account for the expansion of mature macrophages.

HOMEOSTASIS

Different strategies based on the proliferation of YS- or HSC-derived cells or monocyte influx are used by macrophages to maintain their population in peripheral tissues (Figure 2). It has been clearly shown that monocytes are involved in the control of homeostasis[35,36]. Experiments using Cre-loxP-based fate mapping methods or parabiotic mice with mice lacking or not CCR2 have shown that circulating monocytes have a minimal contribution to the maintenance of tissue macrophages in the absence of injury[37,38]. Nevertheless, the sites in contact with microorganisms such as the intestine, skin and spleen are specialized areas in which monocyte input is necessary to maintain macrophage population. Exposure to commensal microorganisms is likely to cause a low grade inflammation also called "primed homeostasis", which is reminiscent of the recruitment of classical monocytes in fully inflammatory conditions[39]. This seducing hypothesis accounts in part for the homeostatic maintenance of macrophage populations such as intestinal macrophages. In mice, the colons of newborns contain macrophages of embryonic origin (F4/80^{hi}CD11b^{lo}) and hematopoietic origin (F4/80^{lo}CD11b^{hi}); the embryonic population of macrophages is prominent after birth and dramatically decreases thereafter[40]. Although macrophages in adult mice retain the ability to divide locally, this ability is not sufficient to account for maintaining macrophage populations in the intestine.

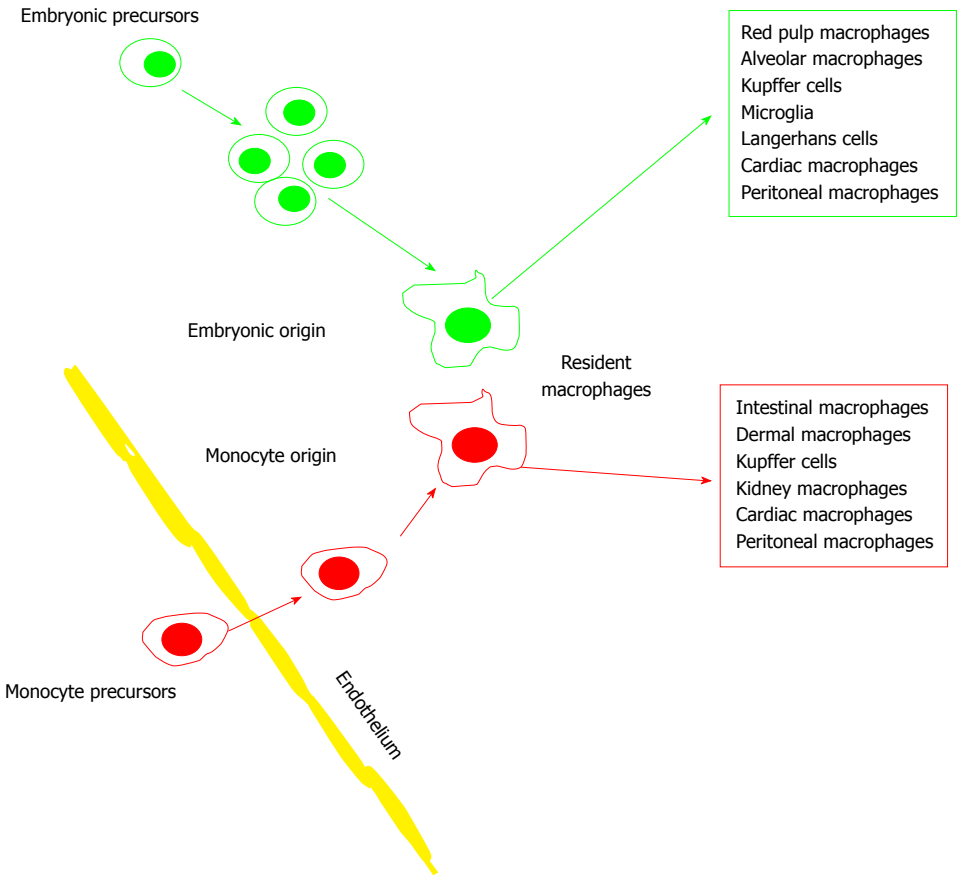

Figure 2 Macrophage origin and homeostasis.

Different studies based on CX_3CR1^{+GFP} mice, the irreversible expression of YPC by $CSF1R^+$ and parabiotic mice demonstrated that intestinal macrophages require constant replenishment through CCR2-dependent recruitment of $LY6C^{hi}$ monocytes[41]. It has also been demonstrated that the constant replenishment of intestine macrophages is related to the microbiota.

Indeed, the administration of broad-spectrum antibiotics for 2 wk in conventional mice results in a small reduction of $LY6C^{hi}$ MHC^+ macrophages but not in the number of $LY6C^{lo}$ MHC^+ mature macrophages in the colon. Hence, homeostasis of resident intestinal macrophages requires both the microbiota and the CCR2-dependent recruitment of inflammatory macrophages.

In contrast, the microglia cells are monocyte-independent. They are localized in the central nervous system and exhibit different morphologies according to the type of activation[42,43]. After birth, the massive expansion of microglia cells that is observed is related to the *in situ* cell proliferation induced by M-CSF and IL-4, but not to monocyte input[44]. The microglia deletion results in decreased synaptic formation and learning[18]. Alveolar macrophages are derived from fetal monocytes that colonize the lungs shortly after birth in a process dependent on GM-CSF and peroxisome proliferator-activated receptor (PPAR)-γ. Another example of macrophages that do not result from monocyte influx is provided by alveolar macrophages. They do not differentiate from blood monocytes because CD163-DTR-mediated depletion results in repopulation by *in situ* proliferation[37,45]. However, when alveolar macrophages are depleted by a genotoxic injury such as a lethal irradiation, recruited monocytes can repopulate the alveolar macrophage niche[18]. Hence, alveolar macrophages do not require monocyte recruitment but can accommodate such requirement when required.

The skin, which has been well studied in mice in recent years, provides an excellent model of the role of the balance between monocyte input and self-renewal. It contains two major macrophage populations, Langerhans cells in the epidermidis and dermal macrophages. The Langerhans cells originate from YS-derived myeloid precursors and monocytes from fetal liver. They undergo an extensive proliferation after birth and a low rate of *in situ* proliferation that is sufficient to maintain their number in adulthood without further monocyte input. Dermal macrophages probably have a complex origin; they contain a pool of established prenatal macrophages and one postnatal pool derived from blood monocytes expressing high levels of CCR2 and LY6C. The dermal macrophages are clearly distinct from Langerhans cells and other dermal DCs[46].

Recent studies concern cardiac macrophages in which depletion experiments enable the description of repopulation strategies. There is evidence that

combined mechanisms are required. In steady state, the majority of cardiac macrophages are of embryonic origin and repopulation after depletion is supported by circulating monocytes[18]. All these findings have been based on murine models and their extrapolation to homeostasis of human macrophages will require original methodological approaches.

MACROPHAGE IN DISEASES

It is well known that macrophages are necessary to fight against microbial pathogens or tumor cells, but they may also contribute to inflammatory and autoimmune diseases and the development of metastasis. While there is evidence that the self-renewal of tissue macrophages seems sufficient to maintain resident cells in steady state, pathological situations require exogenous contribution. Different models of response to injury have provided essential information. The most usual model consists of the injection of microbial or toxic agents inside the mouse peritoneal cavity in which resident macrophages are of embryonic origin. The initial response consists of the recruitment of blood monocytes. During the phase of resolution of inflammation, recruited monocytes mature into macrophages[38]. In a murine model of acute liver injury induced by N-acetyl-p-aminophenol, the number of resident Kupffer cells first decreases and then starts to increase without requiring LY6C^{hi} monocytes during the resolution phase. In the necroinflammatory phase, LY6C^{hi} monocytes are recruited in a CCR2-dependent manner. In addition, the transcriptional signatures of self-renewed Kupffer cells and recruited monocytes are clearly distinct[38]. In patients, some examples illustrate the fact that monocyte influx does not explain the response to inflammatory challenge. For instance, the macrophages present in ocular adnexae are polarized and express markers of proliferative activity[47]. During the acute inflammatory response in which inflammatory macrophages are recruited, tissue macrophages proliferate intensively, suggesting a combination of mechanisms to restore homeostasis after an aggression[48]. We will briefly describe macrophage renewal in some clinical situations.

Atherosclerosis

Atherosclerosis is characterized by the accumulation of macrophages in atheromatous plaques; they ingest lipids and produce a panel of inflammatory mediators leading to an amplification loop. It has been shown at least in mouse models that lesional macrophages arise predominantly from circulating LY6C^{hi} monocytes[49]. The introduction of cholesterol in the diet induces an influx of monocytes in atheromatous plaques after two weeks with a difference between monocyte subsets: monocytes expressing LY6C are more efficiently recruited than monocytes which do not express LY6C and it is believed that the latter cells may promote vascular stability and play an atheroprotective role[50]. Recent papers suggest that the accumulation of macrophages in atheromatous lesions is not the only consequence of monocyte input. Hence, macrophage proliferation in atheromatous lesions has been reported in humans, rabbits and mice[50-52]. In apolipoprotein E-deficient mice (ApoE$^{-/-}$) with a high-cholesterol diet, a rapid turnover of macrophages is observed within the lesions. The monocyte depletion does not affect the turnover of lesion aortic macrophages. In addition, using parabiosis reveals that monocyte recruitment cannot fully account for lesional macrophage accumulation in established atherosclerosis but it cannot be excluded monocyte circulating precursor is involved[50,53]. Using an adoptive transfer methodology in ApoE$^{-/-}$ mice under a high-cholesterol diet shows that proliferating aortic macrophages derive from non-proliferating circulating monocytes. The contribution of recruited monocytes seems to be prominent in early lesions, but it is likely that less than 20% of macrophages in established atheromatous lesions are due to monocyte influx, with local proliferation of macrophages accounting for the largest part of lesional macrophages[50]. Recently, the expression of type 1 scavenger receptor class A (Msr1) on lesional macrophages has been reported and seems to be correlated with macrophage proliferation. Hence, lesional macrophages from Msr1$^{-/-}$ mice proliferate poorly compared with wild type macrophages and are less abundant[53].

The persistence of lesional macrophages also reflects defective cell death. Hence, the lack of macrophage death at early stages of atherosclerosis increases macrophage burden and seems to reduce the progression of the disease in later stages[50,54].

Myocardial infarction is a complication of atherosclerosis and the heart lesion is characterized by an inflammatory response mediated by recruited neutrophils and monocytes, and the proliferation of local cardiac macrophages[55].The expansion of local macrophages is long lasting until healing and these macrophages display heterogeneity of activation states from M1 to reparative M2 macrophages. Healing requires cardiac macrophages whatever their origin (monocyte recruitment *vs* local proliferation) as assessed by numerous studies including clinical studies[56,57].

Recently, it has been reported that Osmr$^{-/-}$ (oncostatin M receptor) mice undergoing myocardial infarction exhibit a reduced number of myeloid cells expressing F4/80 and CD11b. In addition, Osm induces the expression of REG3β, which is a potent chemoattractant for macrophages. In REG3$^{-/-}$ mice with myocardial infarction, the macrophage burden is decreased, suggesting that the macrophages within infarcted lesions are of monocytic origin. Again, the functional activity of lesional macrophages is time dependent. Indeed, the monocytes recruited early within infarcted lesions lead to M1 macrophages whereas macrophages found during tissue remodeling are of M2 type, but these two macrophage functional subsets are controlled by REG3[58]. The inflammatory reaction seems to be similar

in the brain after a stroke. It is likely that the perivascular macrophages replenished by circulating monocytes, in contrast to microglia, drive the recruitment of inflammatory cells in lesions of cerebral tissue. In contrast, microglia may play a role in post-ischemic inflammation and also in tissue repair[59]. Finally, another feature of atherosclerosis is its association with obesity in which macrophages accumulate in adipose tissue and it has been recently reported that local macrophage proliferation is related to obesity-associated adipose tissue inflammation[60].

Cancer

The abundance of tumor-associated macrophages (TAMs) in solid tumors is often correlated with the prognosis of the tumors[9,61]. TAMs are usually of M2 type and may be considered to be repairing the cancer lesions, but the acquisition of tumorigenic properties may involve a complex dialogue between macrophages, tumor cells and stromal cells[62]. The pool of TAMs results from circulating monocytes. Monocyte recruitment depends on the tumor microenvironment and occurs mainly in hypoxic regions of the tumors[63], as demonstrated by different murine tumor models. For instance, in lung adenocarcinoma, two populations of TAMs designated MHCII^{lo} and MHCII^{hi} are present and derive from LY6C^{hi} monocytes[64]. In addition, the spleen is a reservoir for TAM precursors in a CCR2-dependent way[65]. Besides the monocyte origin of TAMs, there is growing evidence that they may also result from a self-renewal process of *in situ* macrophages. It has been reported that TAMs proliferate in human breast carcinomas[66]. Fully differentiated macrophages and not blood-borne precursors drive TAM accumulation in a mouse model of spontaneous mammary carcinogenesis[67]. The situation seems more complex in gliomas in which microglia and TAMs derived from monocytes are present within and around the tumor cells.

Although they exhibit a M2 phenotype under the influence of glioma cells, their origin remains to be determined[43]. It has also been shown that cancer can promote extra-medullary monocytopoiesis in spleen red pulp. In murine lung adenocarcinoma, angiotensin is directly involved in the self-renewal of HSCs and macrophage progenitors; the blockade of its production restrains the number of TAMs[68]. In cancer, the recruitment of monocytes seems to be prominent but understanding of the nature of the dialogue between macrophages and tumoral cells is only in the early stages.

Infectious diseases

The recruitment of monocytes and their maturation in macrophages is essential for defense against microbial pathogens[5]. Indeed, monocytes enter sites of infection and draining lymph nodes to promote adaptive immunity. For instance, the recruitment of inflammatory monocytes in the lungs in response to *Mycobacterium tuberculosis* is necessary to T-cell activation and tuberculosis control[69]. In several infectious diseases, CCR2-mediated monocyte mobilization plays a prominent role. The recruitment of monocytes mediated by CCR2 is required for the control of *Legionella pneumophila* infection in mice[70]. The protective role of LY6C^{hi}monocytes *via* the CCR2 pathway has been reported in infections with *Plasmodium chabaudi* and *Cryptococcus neoformans*[5]. Most of these inflammatory monocytes mature in tissue lesions and granulomas into macrophages and DCs, and exhibit a M1 profile[71]. Cytomegalovirus (CMV) infection is known to reprogram monocytes towards a M1 phenotype[72]. In a mouse model of congenital CMV infection, the virus is responsible for neurological lesions, disruption of the self-renewal of neural stem/progenitor cells and increased number of activated macrophages (meningeal macrophages and parenchyma microglia) in infection foci. The increased macrophage infiltration may be due to the recruitment of macrophage precursors[73].

Besides the prominent mechanism related to monocyte influx, M2-polarized macrophages are associated with the self-renewal of macrophages in tissues in some parasitic infections. In experimental filariasis, *Litomosoides sigmodontis* worms are killed in the pleural cavity in resistant C57BL/6 mice; the depletion of blood monocytes does not prevent the expansion of macrophages in the pleural cavity of infected mice. The expansion of resident macrophages can be mimicked by the administration of IL-4[30]. Nevertheless, M2 macrophages in the intestinal tract of nematode-infected mice are largely monocyte-derived and the macrophages of lamina propria from these mice are able to proliferate, thus demonstrating the complexity of macrophage origin in helminth infections[29]. It will be important to determine if the self-renewal of macrophages is only a property of helminthiasis infections and what the role of this property of human infectious diseases is.

Neurodegenerative diseases

The pathogenesis of neurodegenerative diseases is critically associated with the neuroinflammation that involves several cell types including microglia. The blood-brain barrier slows down the traffic of monocytes from the blood to the central nervous system and has to be integrated to understand neuroinflammation. Parabiosis and BM transplant studies in mice have revealed the infiltration of monocytes in experimental autoimmune encephalomyelitis and that this infiltration is related to the progression of the disease and the breakdown of the blood-brain barrier. The recruited monocytes are eliminated over time whereas microglia cells expand locally through proliferation in a persistent manner[36,74]. While monocyte-derived macrophages may be responsible for demyelination, microglia maybe involved in clearance of cellular debris[17]. Alzheimer disease is characterized by the deposition of amyloid-β

into parenchyma, the formation of neurofibrillary tangles and neuroinflammation.

Although there is no overall change in microglia cell numbers in the late stages of Alzheimer disease, the chronic stimulation of microglia may result in microglia loss and further replenishment within the brain in the early stages of the disease through the proliferation of tissue-resident microglia[75]. IL-34 induces the proliferation of microglia which results in the clearance of soluble oligomeric amyloid-β; co-cultivating primary neurons with microglia in the presence of IL-34 attenuates the neurotoxicity of amyloid-β. The protective effect of IL-34 has been observed in a mouse model of Alzheimer disease in which IL-34 is administered in intra-cerebral ventricles[76]. Alternatively, macrophages of BM origin may also contribute to Alzheimer disease pathogenesis. It has been proposed that self-renewing microglia produce chemoattractants that may also attract myeloid cells to neuroinflammation sites[42]. Other studies have reported that CCR2-expressing macrophages are the preferential population recruited to amyloid-β deposits and CCR2 deficiency is associated with impaired amyloid-β clearance[77]. Finally, Huntington disease is due to the expansion of the trinucleotide CAG in the gene encoding huntingtin, which is associated with microglial activation. A proliferation of microglia seems to be critical in the pathogenesis of the disease. Indeed, experiments based on cultures of microglia and brain slices have revealed the activation of microglia and their proliferation in the vicinity of degenerating neurons expressing mutated huntingtin[78].

CONCLUSION

The introduction of new molecular tools has greatly modified our vision of the origin of macrophages. The role of circulating monocytes in replenishing macrophage populations seems to be limited in steady state conditions even if they play an important role in pathological conditions. The demonstration of mature macrophages' ability to proliferate has profoundly changed our vision that this proliferation reflects macrophage immaturity. The proliferation of macrophages seems to be associated with macrophage polarization in pathological conditions. These new results open fascinating perspectives in different pathologies. The interference with the recruitment of monocytes with therapeutic monoclonal antibodies is already a means to modify the microenvironment of tumors for instance, but this strategy may be a source of potential complications including infectious complications. Better knowledge of the origin of macrophages in lesions may lead to the reprogramming of macrophages to enhance their beneficial functional properties without promoting deleterious effects.

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MINIREVIEWS

Helicobacter pylori vs immune system or antibiotics

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Abstract

Helicobacter pylori (*H. pylori*) infection has often no clinical signs and is one of the most common bacterial infections. All infected subjects have histology of active chronic gastritis. In some cases patients develop peptic ulcer and minority of them develop gastric cancer. Gastric cancer is multifactorial disease, thus various progressions of *H. pylori* infection and disease are dependent on the host genetic factors, the characteristics of the individual's immune response, environmental factors, and different bacterial virulence factors of the individual bacterial strains. Eradication of the bacteria plays a crucial role in the treatment of these cases however antibiotic therapy does not always help. Bacteria often develop resistance to antibiotics so we recommend that not only screening for *H. pylori* also the strain determination should have some diagnostic value, especially in the patients who already developed gastritis. Furthermore, for such patients assessment of disease progression (atrophic or metaplastic gastritis) could be followed by polymorphism determination. Until now we cannot predict the disease based only on single polymorphism. Bacteria successfully neutralize the responses of the immune systems using different enzymes or even components of the host immune response. However, the influence of immune system and its components could represent new ways of treatments and could help to eradicate the infection.

Key words: *Helicobacter pylori*; Resistance to antibiotics; Immune response; Genetic factors; Bacterial eradication

Core tip: Combination of *Helicobacter pylori* (*H. pylori*) and host-associated risk factors do not always allow evaluation of gastric carcinoma. We have learnt that the assessment of patients with *H. pylori* infection and its strain is very important and concluded that eradication of bacteria has essential meaning. We recommend that not only screening for *H. pylori* also the strain determination should have some diagnostic value, especially in the patients who already developed gastritis. Furthermore, for such patients assessment of disease progression could be followed by polymorphism determination. Conclusions indicate that host cytokine genotypes, host immune response, as well as *H. pylori* strains could be important for greater risk for developing gastric cancer.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is 3-5 μm Gram-negative microaerophilic bacterium. It colonizes the gastric mucosa and metaplastic gastric epithelium of duodenum. It has characteristic spiral morphology with more flagella at one pole and is capable of moving in the protective gastric mucus layer. It has enzymes catalase, oxidase and degrades urea. While living in extremely unfavorable environment of low pH in the stomach, the protection for survival is provided by the clearance of gastric mucus and secretion of proteases that degrade additional mucus, and urease that produces ammonia, which helps to raise the pH in the vicinity of bacteria[1,2].

Epidemiology

H. pylori infection results in chronic active gastritis, and 20% of infected also develop ulcers of the stomach or duodenum. In some cases infection leads to stomach cancer and MALT lymphoma. Although *H. pylori* is not invasive bacteria, the mechanisms of mucosal inflammation and tissue damage and the onset of the disease are a combination of bacterial and host factors[2]. *H. pylori* is a human pathogen, and its reservoir is the human stomach. It colonizes cardio, corpus and antrum of the stomach. The transmission of *H. pylori* from the stomach of one human to another is not yet fully clear. The transfer is most common within families and during childhood. In all likelihood, there are two routes of transmission; the oral-oral route and the fecal-oral route. Fecal-oral route is associated with the ability of bacteria to survive outside its primary environment. For this pathway certain conditions must be met, namely the rapid passage through the intestine, most often in the case of diarrhea, inadequate sanitation and sewage disorder. Furthermore, contamination of drinking water by sewage is also possible. Such conditions are usually found in developing countries where the prevalence of *H. pylori* infection is greater than in developed countries. Oral-oral route is associated with regurgitation of gastric juice and thus passage of bacteria in the mouth and the transmission of *H. pylori* in close contact to another person[3,4]. *H. pylori* infect more than half of the world's population. This bacterium can be left in the gastric mucosa for a long time, without causing disease symptoms including rare spontaneous disappearance from the stomach[3]. The highest infection rate is 54% of the age group 60 to 69 years. In the age group 0-19 years, 10% of those living are infected, and in the age group between 20 and 49 years 29% of the population is infected with *H. pylori*[5].

The pathogenesis and virulence factors

H. pylori has adapted to live between the protective layer of mucus and epithelial cells of the gastric mucosa. The bacterium develops in neutral pH, and is changed to coccoid shape at a pH below 4 or above 8. The gastric mucosa can be damaged directly or indirectly by influencing on the homeostasis of acid secretion[6].

The inner part of the *H. pylori* cell membrane, which is situated at the cytoplasmic membrane, consists of peptidoglycan. On the outer side the outside membrane with anchored lipoproteins is located, which are sometimes covalently bound to the peptidoglycan. The main surface components of Gram-negative bacteria are lipopolysaccharides (LPS), which protect the bacteria from the environment[3]. The basis represents the lipid A of LPS, which allows the biological activity of the endotoxin[3,7]. *H. pylori* has enzyme urease on the outer and inner membrane, which can be excreted in the neighborhood through the membranes of dead cells. It degrades urea to ammonia and bicarbonate, and thus neutralizes the acid environment. The bacterium is able to attach on epithelial cells of the gastric mucosa and moves as a spiral through the mucus in the stomach. The enzymes protease, lipase and phospholipase allow to cleavage the glycoprotein of mucous gel into a more hydrophobic structure, which can cause diffusion of hydrogen ions and possible damage to the mucosa. Ammonia can react with hypochlorous acid of neutrophils resulting in cytotoxic compounds such as monochloramine and hydroxylamine[7].

Genetic information for vacuolization cytotoxin (Vac A) have all *H. pylori*, approximately 50% of them produce 67 kDa protein, which causes the formation of vacuoles in epithelial cells of the gastric mucosa. Cytotoxin-associated gene A (*Cag A*) is the genetic makeup of the 127 kDa protein, which is present in all strains. Bacteria that produce CagA cause pronounced secretion of IL-8 from epithelial cells, resulting in increased mobilization of neutrophils and a higher level of active chronic gastritis. Neutrophils cannot effectively phagocyte bacteria and at quicker rupture of the neutrophils free oxygen radicals are released, which further failures the mucous membrane. Patients infected with Cag A-positive strains have increased risk for the development of duodenal ulcers and gastric cancer[6,8].

H. pylori has a protein which causes the attachment of neutrophils to the endothelial cell lining of the stomach and is called HP-NAP (*H. pylori* neutrophil-activating protein). Different degrees of attachment of neutrophils were observed, which are caused by different activities of this protein in various strains of *H. pylori*, which indicates different levels of expression of this protein as similar to protein Vac A. HP-NAP is located in the cytosol of bacteria and is released during lysis. It binds to the outer surface of the outer membrane, similar as urease. The majority of patients infected with *H. pylori* produce a specific antibody to HP-NAP. Vaccination of mice with the protein HP-

NAP provided protection against infection with *H. pylori*. Protein plays an important role in the immune response to infection with the bacterium. HP-NAP is chemotactic for monocytes and neutrophils, and causes increased expression of β2-integrins on neutrophils and monocytes. HP-NAP works through activation of NADPH oxidase in neutrophils and thereby induces the production of reactive oxygen intermediates Reactive oxygen intermediates. HP-NAP represents a virulence factor that is associated with the pathogenic effects of the *H. pylori* to the site of infection[9].

H. pylori affect the homeostasis of gastric acid secretion through changes in paracrine hormone control system for gastrin and acid secretion. Normally, the release of gastrin leads to acid secretion and lowering the pH below 3 promoting cells D to somatostatin secretion, which in paracrine route inhibits the release of gastrin, and consequently acid secretion. Proteins and fatty food in the duodenum *via* cholecystokinin stimulate the secretion of somatostatin and thereby inhibit further secretion of acid. Patients with duodenal ulcer and infected with *H. pylori*, have increased sensitivity of the parietal cells to gastrin. These patients, when stimulated with food or with gastrin, secrete larger amounts of stomach acid than healthy people. After removal of bacteria, secretion of gastrin normalizes, the amount of acid is reduced by 50%, and completely normalizes only after one year. Infected patients have also reduced secretion of bicarbonate into the duodenum. After removal of the bacteria this is also normalized[6].

Virulence factors CagA and VacA and the development of gastric cancer

Virulence factors CagA and VacA are the most important factors of *H. pylori* in the development of the disease. The CAG-pathogenetic islands (cagPAI) have the information for the type Ⅳ secretory system, which is required for peptidoglycan to enter the cell. CagA phosphorylates cellular proteins, which are known oncogenes[10]. The motive for the phosphorylation of CagA is located within the amino acid motif EPIYA. A larger number of EPIYA-C within CagA damage more cells and more severe course of the disease occurs, especially gastric cancer[11]. CagA after phosphorylation connects to the proteins in the cell, which causes an increase in signal similar to that in the expression of growth factors in the cell. It affects the proliferation and adhesion of cells and the organization of the cytoskeleton[12].

VacA is a protein that forms pores in the cell and causes vacuolization and cell death. It is involved in the presentation of antigen and causes efflux of ions out of the cell due to the impact on the integrity of close connections between cells (tight junction). It is also a potent inhibitor of cell activation of T-lymphocytes *in vitro*[13,14]. *H. pylori* has several versions of the protein VacA. Type VacA s1/m1 is the most cytotoxic. Interesting findings are that CagA positive strains has vast majority of s1 type VacA. Vac A has two versions, i1 and i2. Protein VacA s1/m1 i1 is often present in patients with gastric cancer[15].

The immune response to infection

Chronic gastritis is associated with increased local production of IgG, increased infiltration of the mucous membrane with T cells, and increased expression of HLA class Ⅱ molecules[16,17]. In addition, chronic gastritis is a *H. pylori* infection associated with other diseases, such as acute gastritis, peptic ulcer on duodenum, lymphoma of gastric mucosa and gastric adenocarcinoma, which is divided into intestinal and less common diffuse type[18]. The development of disease stages can be divided into three entities. Simple gastritis caused by infection with bacteria, acid secretion is normal. Duodenal ulcer occurs when infection with the bacterium occurs, antral gastritis is present, and acid secretion is increased. This type of patient is protected from gastric cancer. *H. pylori* chronic gastritis in patients with gastric cancer is more intense in the corpus, also more extensive intestinal metaplasia and atrophy of the mucosa are present. There is not enough acid, so intestinal metaplasia might develop, which leads to the intestinal-type of gastric cancer[19,20].

Infection with *H. pylori* causes an increased concentration of IL-8, IL-1, IL-6 and TNF-α. IL-8 in tissue, which activates neutrophils, results in the epithelial cells, as well as in other cells in the wall of the stomach. Expression is dependent on the adherence and the genotype of the *cagA*. The immune response Th1 is triggered in case of infection by intracellular pathogens and cancer, immune responses Th2 is characterized in infection by extracellular pathogens. In contrast to the expected reaction in the host with *H. pylori* infection, Th1 immune response is particular. Bacteria should choose an immune response by stimulating IL-12, which leads to a Th1 response. Thus stimulated immune system cannot overcome the infection. The cellular immune response and the formation of IgG antibodies which can activate complement cause intense inflammation and further damage to the mucosa. Neutrophils which are attracted and activated by IL-8 are an important component in the development of chronic active gastritis. The result of infection with the same strain of *H. pylori* could be different in the immune response of the different entities[6,21].

Infection of the gastric mucosa, accompanied by a strong neutrophilic infiltration of the mucosa, significantly contributes to the formation of gastritis. Strains of *H. pylori*, which are capable to activate neutrophils, are more common in patients with peptic ulcer than in patients with chronic active gastritis[9].

H. pylori possess number of factors that contribute to the colonization and to bacterial adherence. BabA binds to antigens of Lewis b blood group, which are on the cells of the epithelium, and can contribute to the adhesion of bacteria. Transgenic mice expressing

more Lewis b blood group have had heavier chronic gastritis and have lost several parietal cells[4]. SabA is a protein on the outer membrane of *H. pylori* and binds to a glycoprotein antigen of Lewis x blood group and facilitates adhesion. Similar role has OipA. OipA is on the membrane of *H. pylori* is more pronounced in people who already have precancerous changes. Bacteria survive easier in the epithelial cells, since it is difficult to mechanically removed[22].

The influence of immune system on the success of treatment of H. pylori infection

In addition to *H. pylori* for the development of the disease we must also consider the immune system. T lymphocytes in the gastric mucosa mostly express IFN-γ, which indicates Th1 immune response[23]. In patients who have weakened immune response despite treatment with antibiotics are not healed. T lymphocytes without additional stimulation with antigens of *H. pylori* produce smaller amounts of IL-4 than in patients whose therapy was successful with antibiotics[24]. In the patients who received antibiotic therapy and are cured, T lymphocytes, stimulated by antigens of *H. pylori*, express larger amounts of IFN-γ molecules compared to the patients who do not recover despite receiving treatment against bacteria. In cured patients compared with those which were not cured, an increased expression of IFN-γ and IL-4 was observed, when T-lymphocytes were stimulated with dendritic cells, which have been in contact with *H. pylori* antigen[25].

One of the possible methods for treatment of *H. pylori* infection could be *in vivo* addition of recombinant IFN-γ. In normal humans, the addition of IFN-γ mimics the physiological response to bacterial infection, causing increased expression of FcγRI, which is proportional to the biological activity of IFN-γ on neutrophils and monocytes, which enhances phagocytosis by neutrophils mediated by FcγR. In addition to improved defense in normal humans, recombinant IFN-γ also helps in treating various disorders of immunity[26].

It was also found that IL-12 produced by the antigen-presenting cells (APC), in the *H. pylori* infection effected naive CD4-T-lymphocytes, which are directed towards a Th1 response through the transcription factor T-bet and STAT4. This suggests that the infection with *H. pylori* is an important stimulus for the secretion of IL-12[27].

Moreover, IL-18 affects the production of IFN-γ released by T-lymphocytes and NK cells. If the mucosa is infected with *H. pylori*, the regulation of IL-18 expression is influenced by CagA and OipA. Epithelial cells produce IL-18 under the influence of two virulence factors, while monocytes produce more IL-18 only under the influence of OipA. These data confirm the importance of IL-18 in the development of gastritis due to *H. pylori* infection[28]. Th17 T cells could also play an important role in infection with *H. pylori*. It is anticipated that IL-17 plays an important role in mucosal immunity. The expression of IL-17 in the case of *H. pylori* infection is affected by IL-23. If this path is blocked, the infected mucosa has less IL-17[29,30].

APCs have on their membranes' receptors that recognize PRR (Pattern Recognition Receptors), which include Toll-like receptors (TLR). These are specific receptors, which are involved in the mechanisms of innate immunity and can trigger a series of defense mechanisms, such as complement activation, phagocytosis and expression of genes associated with inflammation. TLR receptors recognize conserved structure of microorganism, which are relatively stable within certain groups of microbes and are called Pathogen-Associated Molecular Pattern or PAMP[31]. TLRs recognize different molecular components of microorganisms. Examples of ligands are LPS from the cell wall of Gram-negative bacteria, peptidoglycan, lipoproteins and lipopeptide from the cell wall of Gram-positive bacteria. TLRs detect some bacterial proteins such as flagellin, and a foreign nucleic acid. LPS was the first detected ligand for the TLR, which is recognized by TLR-4. TLR-4 is part of the lipopolysaccharide receptor CD14, and for recognition binding of the complex of LPS and LPS binding protein (LBP) to the receptor CD14 is required[32,33].

On the surface of the membrane of Gram-negative bacteria are LPS, which protect bacteria against bile salts, hydrophobic antibiotics and complement activation. LPS after release organize in aggregates. Using protein LBP a complex LPS-LBP is formed. This binds to the membrane protein CD14 (mCD14), which is located on monocytes, or a soluble CD14 protein (sCD14) which is present free in the serum[31]. LPS of *H. pylori* (Hp LPS) is, compared to the other Gram-negative bacteria, poor immunogen. In addition, Hp LPS as compared to other bacteria binds poorly to the TLR-4 on epithelial cells. TLR-2 and TLR-5 are more important for the innate immune response of epithelial cells on *H. pylori*[34,35]. Due to the poor immunogenicity of Hp LPS scientists began to look for other receptors, which participated in the initial process of the immune response. They found that an important role play *H. pylori* peptidoglycans, which are an important link in the development of mucosal immunity. Peptidoglycan enters the epithelial cell using the type Ⅳ secretory system, which genetic information is located in cagPAI of *H. pylori*. Inside the cell it binds to the NOD-like receptor, which ultimately increases the kinase activity of NF-κB, which activates cell proliferation, and then through the other signals the activation of the immune system[35].

Dendritic cells (DC) play an important role in directing the immune response. *H. pylori* is presented to other immune cells, especially T lymphocytes, and is involved in their activation. In what way DC focus the immune response is not yet fully understood. We know that the response of DC depends on the virulence factors of *H. pylori* and the host immune competence[16]. DC can direct differentiation of T lymphocytes into Th1 subsets, with the consequent emergence of severe gastritis, or subsets Th2, which causes lighter inflammation. Th2 response, in the case of *H. pylori* infection, is less

frequent than Th1. The differentiation in regulatory T-lymphocytes is also possible, which limits the immune response and thereby prevents the formation of more severe forms of inflammation, what helps *H. pylori* to survive[17,36]. DCs which present antigens of *H. pylori*, strongly activate T-lymphocytes, influence on the production of cytokines and initiate an inflammatory process. *H. pylori* LPS (Hp LPS) stimulate DC *via* TLR[37]. TLR are less important for the immune response in the case of epithelial cells, however the APC use TLR for the immune response[38]. DC, together with the cytokines and costimulatory molecules, affect other inflammatory cells, especially the T lymphocytes. Measurement of the immune response in case of infection with *H. pylori* could help to assume in which patients standard therapy with antibiotics are more likely to be ineffective[25].

The influence of host factors on the development of gastric cancer

Several host genetic factors are important for the progression and development of gastric cancer. Single nucleotide polymorphisms or point mutations in genes for cytokines affect gastric acid secretion and innate immune response[39-42]. Polymorphisms in genes may influence the level of the cytokine production, and consequently influence the disease outcome[43].

IL-1β is mainly secreted in response to *H. pylori* infection. It has a proinflammatory activity and strongly inhibits gastric acid secretion[44]. Inhibition of acid secretion leads to the spread of bacteria from the antrum to the corpus, and consequently the development of corpus predominant gastritis which further leads to the development of gastric cancer[45,46]. Three polymorphisms were described in the *IL-1B* gene at positions -31, -511 and +3954 from the transcription start site[45,47]. *IL-1B*-31*C and *IL-1B*-511*T alleles are associated with hypochlohydria or decreased acidity in the stomach in response to the *H. pylori* infection[45]. IL-1β receptor antagonist polymorphism (IL-1ra) has also been associated with the level of IL-1β secretion. Genotype *IL-1RN**2 is associated with higher secretion of IL-1β, most probably through the reduction of its receptor antagonist IL-1ra[47,48].

TNF-α as a central mediator of the immune response has several polymorphisms in the promoter region of *TNF-A* gene of which -308*G > A was associated with increased production of TNF-α in response to the infection, and increased risk of gastric cancer[49-51]. El-Omar *et al*[52] and Machado *et al*[53] found that patients with this polymorphism have almost two-fold increased risk of gastric cancer.

At the position +896, in exon 4 of the *TLR-4* gene a functional polymorphism has been described. This A > G transition results in an alteration of the extracellular domain of TLR-4, that causes hyporesponsiveness to LPS, reduced epithelial TLR-4 density and exaggerated inflammatory cytokine response[54]. A recent studies have reported an association of *TLR-4* gene polymorphisms with gastroduodenal diseases such as gastric atrophy, hypochlorhydria and noncardia gastric cancer[55-58].

Furthermore, our results on Slovenian population showed that males were more predominant to develop gastric cancer than females. Meanwhile females had 2-fold greater probability to develop chronic gastritis[59]. We also proved that *IL-1B*-511*C homozygote allele was most frequent in chronic gastritis group (58.8%). Such results were not found in any other study. According to our findings, individuals carrying the *IL*-1B-511*T/T allele, both homozygotes and heterozygotes, compared to control group showed an increased OR for gastric cancer. Moreover, no indications that the infection with *H. pylori* in a given inflammatory genotype could result in an inflammatory response, and then gastritis or cancer could be found[59]. *TLR-4* or *TNF-A* polymorphism did not play a role in the development of gastric premalignancies. The results were comparable to those by Garza-Gonzales *et al*[55] and confirmed in review by Figueiredo *et al*[60] for TLR-4. Meanwhile, in 2015 Trejo-de la *et al*[61] suggested that 2848G > A polymorphism in *TLR-9* increased the risk for the development of duodenal ulcer.

Treatment of H. pylori infection

In addition to the immune response, which is difficult to influence, an appropriate antibiotic therapy is important. Infection with *H. pylori* is most effectively cured with a proton pump inhibitor (PPI) and a combination of two antibiotics. We mainly use metronidazole and clarithromycin or clarithromycin and amoxicillin or amoxicillin and metronidazole. Certain strains of *H. pylori* became resistant to metronidazole and clarithromycin. However, with antibiotics we cannot eradicate *H. pylori* in about 10% of patients in whom the bacterium is not resistant to selected antibiotics[62-64]. If we want to successfully treat the *H. pylori* infection, we need to know the primary resistance of *H. pylori* to antibiotics. Less developed regions have a very high resistance to clarithromycin and metronidazole, in the case of metronidazole ranging up to 100%[64]. Indications for treatment and methods of treatment are set out in national and international guidelines. The success of the treatment decreases with age, and the 7-d treatment in the United States is between 57% and 73%. The reasons for the decline in the performance of treatment are mainly the creation of resistance of *H. pylori* to antibiotics and poor patient compliance with the treatment[65-67]. In Slovenia the performance of traditional 7-d treatment regimen OMC (proton pump inhibitor such as PPIs, omeprazole 2 × standard dose, metronidazole 2 × 400 mg, clarithromycin 2 × 250 mg) and OAC (PPIs, 2 × standard dose amoxicillin, 2 × 1000 mg, clarithromycin 2 × 500 mg) was last checked 10 years ago. In 1997 and 1998, the effectiveness of treatment with the scheme OMC was 82.6% and in the group treated with the scheme OAC 82%[68-70].

Globally the decline in the success of the treatment of *H. pylori* infection with a regimen that last 7 d was detected. The success of treatment is between 57% and 73%. In Europe the resistance to clarithromycin is in the range between 1% and 21.3%, metronidazole between 14.4% and 38%. In Slovenia in 2000 *H. pylori* was resistance to clarithromycin 3.7%, and to metronidazole 18.5%[71,72]. It was determined that there is still adequate resistance to clarithromycin between 15 and 20%. When a certain area exceeds this limit, it is necessary to think about replacing clarithromycin with another drug or control the sensitivity of each *H. pylori* isolate and adjust therapy to antibiogram. In 2010 in our group of Slovenian isolates we found 18.6% resistance to metronidazole and 17.5% resistance to clarithromycin. We have found that combined resistance to metronidazole and clarithromycin is 4.1%. Resistance to amoxicillin and tetracycline were not detected. Resistance to ciprofloxacin is 3.1%[73].

It is not always the fault of bacterial resistance to antibiotics for failed eradication. As mentioned above, in about 10% we fail to eradicate bacteria in the stomach, despite adequate sensitivity[72-74], and although patients followed doctor's instructions about taking antibiotics after repeated therapy failed to remove bacteria. With a better understanding of the mechanism of the immune response during infection and treatment we could explain why some patients despite antibiotic therapy do not react appropriately and the problems due to repeated infections persist and do not lead to eradication of bacteria[64,74].

Cathepsins

For this type of protease has long been thought that their application is restricted to the final degradation of proteins in lysosomes, but it was subsequently proved to be involved in several very important cellular processes. It is considered that cathepsins are involved in intra- and extracellular protein decomposition, processing propeptides and hormones, apoptosis, transformation of bone tissue, reproductive processes and the processes of differentiation, in addition, increased motility and invasion in the cells. Cysteine cathepsins are involved in various effector mechanisms of acquired and innate immune response and are essential for an effective immune response. Cathepsins are also indispensable for differentiation, adhesion and migration of immune cells, regulation of cytokines, induction of apoptosis, and many other processes[75]. Disturbed regulation of their enzymatic activity is associated with cancer, and their manipulation is shown as an option for the development of new drugs[76,77]. Cathepsins are important targets for the development of new molecules for the diagnosis, prognosis and therapy of cancer[78,79].

The activity of cathepsins is controlled by regulating the synthesis and processing of cathepsins, inhibition of endogenous inhibitors (stefins and cystatins) and pH stability[80]. Conventional cathepsins are lysosomal enzymes which are only active at acidic pH. This has changed the fact that many cathepsins in physiological and pathological conditions diverted from lysosomes into the extracellular space in other cellular organelles or in the cytoplasm and can be active at neutral pH[81].

Cathepsin X and the immune response to infection with H. pylori

Cathepsin X is a lysosomal cysteine protease located in macrophages gathered from gastric mucosa. Patients with *H. pylori* gastritis had a higher concentration of cathepsin X protein and cathepsin X mRNA levels in gastric mucosa compared to *H. pylori* negative patients[82]. Cathepsin X was also up-regulated in the gastric mucosa of patients with gastric cancer in contrast to patients without cancer[83].

We tested if the inhibition of cathepsin X influences the successful immune response to a *H. pylori* infection. We have proved the involvement of cathepsin X in the antigen presentation with TLRs. When THP-1 cells with different strains of *H. pylori* were stimulated, the addition of the inhibitor of cathepsin X resulted in a higher expression of TLR-4 on the membranes of THP-1 cells. This was especially true in clarithromycin sensitive strains of *H. pylori*. The expression of TLR-4 and TLR-2 was significantly higher when *H. pylori* stimulated DCs were cultivated together with cathepsin X inhibitor compared to the dendritic cells stimulated with *H. pylori* only[84].

The influence of higher expression of TLR-4 on the membranes of THP-1 cells on the production of cytokines IL-1b, IL-8, IL-10, and IL-6 was also tested. The concentrations were lower in the group of *H. pylori* strains that were resistant to clarithromycin. The same was seen in the THP-1 cells where we added bacteria along with the inhibitor of cathepsin X. It seems that the inhibition of cathepsin X influences the concentrations of cytokines, as well on the TLRs, that are crucial for efficient regulation of immune response to *H. pylori.* We discovered that strains that are resistant to clarithromycin are less immunogenic than clarithromycin sensitive strains and that they are capable of surviving an immune system attack for a prolonged period of time and as well develop resistance to clarithromycin that further attributes to eradication failure of *H. pylori*[84].

We have proved that resistance to clarithromycin can be a problem for the eradication since such strains seem to be less immunogenic. We assumed that the inhibition of cathepsin X to control the immune response in the cases with impossible eradication of *H. pylori* would not be beneficial. The immune response to infection would be delayed and thus could lead to persistence of bacteria and possible disease progression from atrophy, metaplasia to gastric cancer. On the other hand, when gastric cancer is already developed, inhibition of cathepsin X could be helpful since we could influence the process of cell senescence and also influence tumour cell growth.

CONCLUSION

After this review we can conclude that *H. pylori* are very successful bacteria avoiding host immune response to infection. Furthermore, not only infection itself, but also the immune response is important for the development of gastric cancer. Host cytokine gene polymorphisms represent just one component of complex interactions among host, pathogen, and environmental factors involved in gastric carcinogenesis. Only combination of *H. pylori* and host-associated risk factors do not always allow evaluation of gastric carcinoma. The disease progression from infection through atrophy to neoplastic transformation depends on other factors, including diet and different pathogenesis of *H. pylori* strains. Now we have learnt that the assessment of patients with *H. pylori* infection and its strain is very important and concluded that eradication of bacteria has essential meaning. We recommend that not only screening for *H. pylori* also the strain determination should have some diagnostic value, especially in the patients who already developed gastritis. Furthermore, for such patients assessment of disease progression (atrophic or metaplastic gastritis) could be followed by polymorphism determination. Altogether, conclusions indicate that host cytokine genotypes, host immune response to infection, as well as *H. pylori* strains could be important for greater risk for developing gastric cancer. However, we think those parameters alone could not predict the incidence and risk of the disease, only the combination could be of greater value.

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Lessons from Sjögren's syndrome etiopathogenesis: Novel cellular and molecular targets

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Abstract

Sjögren's syndrome (SS) is a systemic autoimmune disease that affects primarily the lacrimal and salivary glands. In addition to a systemic autoimmune response directed against ubiquitous antigens (such as Ro and La antigens), patients with SS mount a localized response that affects the epithelial component of exocrine glands leading to the establishment of a destructive inflammatory infiltrate comprised of activated T and B cells. Local chemokine and cytokine production drive the recruitment and local activation of immune cells that cause injury to acinar cells. CD4 T cells with different functional differentiation programs including Th1 (IFN-γ), Th2 (IL-13, IL-4) and Th17 (IL-17, IL-21, IL-22) as well as diverse cytokine signaling pathways, are involved at the initiation, perpetuation, and progression of the disease. Which factors initiate this response and allow it to become chronic are unknown. Proposed mechanisms include viral infections and acinar cell apoptosis. Moreover risk-conferring genetic variants, probably through the facilitation of innate and adaptive immune activation, most certainly contribute to the creation of an underlying environment that fosters tolerance loss and facilitates perpetuation of the autoimmune response. In this review, we describe the mechanisms through which the immune response causes SS and emphasize the pathways that are amenable of being targeted with therapeutic purposes.

Key words: Sjögren's syndrome; Pathogenesis; Therapy; T cell; Cytokines

Core tip: Sjögren's syndrome (SS) is a complex entity caused by an autoimmune process that encompasses both an anti-acinar and a systemic response. Exocrine gland infiltration is probably primarily responsible for the destruction of the acinar cells and consequently for the development of sicca symptoms. The participation of diverse chemokines, activated T cells and B cells, cytokines and cytokine signaling pathways has been recognized. The aim of this review is to discuss some aspects of SS pathogenesis and emphasize the potential

opportunities where therapeutic interventions might be useful.

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INTRODUCTION

Primary Sjögren's syndrome (PSS) is a chronic systemic autoimmune disease. Clinical and pathological manifestations in patients with PSS are most evident in exocrine glands, in particular in salivary and lacrimal glands. However, patients frequently also exhibit extraglandular manifestations[1].

The origin of the disease is unknown. An autoimmune response primarily directed towards exocrine glands develops in the midst of a less well defined systemic loss of tolerance. Why some patients with systemic autoimmunity develop a response against lacrimal and salivary glands is still poorly understood, but local factors that induce infectious and non-infectious cellular injury are probably involved. Risk-conferring genetic variants, probably through the facilitation of innate and adaptive immune activation, most certainly contribute to the creation of an underlying environment that fosters tolerance loss and facilitates perpetuation of the autoimmune response upon environmental triggering elements[2].

Sicca symptoms, the hallmark manifestation of Sjögren's syndrome (SS) are the result of acinar cell dysfunction and destruction (Figure 1). Because both phenomena are primarily immune-mediated, the interruption of the anti-acinar immune response has been the focus of most therapeutic proposals. T cells comprise a large fraction of the inflammatory infiltrate of affected salivary glands. Therefore, they are considered important players in the pathogenesis of SS[3]. Other cells, including B cells which undergo local activation and proliferation, also play a central roles in the development of the disease[4].

The aim of this review is to discuss some aspects of SS pathogenesis and emphasize potential opportunities where therapeutic interventions might be useful. As targeting B cells has been recently discussed in the literature[5,6], we have decided to focus in other potential approaches.

SYSTEMIC AUTOIMMUNITY

SS is considered a systemic autoimmune disease, because in contrast to patients with organ-specific autoimmune conditions such as type 1 diabetes, patients with SS mount a robust immune response mostly directed against ubiquitously expressed molecules (*e.g.*, Ro antigen). Amid this systemic autoimmune response, patients with SS develop a response that preferentially affects exocrine glandular tissue, most notably acinar cells from lacrimal and salivary glands. This anti-acinar response is a unifying characteristic, a sine qua non that defines SS present either as a primary disease or as a condition associated to other systemic autoimmune diseases (secondary SS).

The systemic autoimmune response probably precedes and underlies the anti-acinar response. Further, it contributes to the pathogenesis of extraglandular manifestations present in up to 60% of patients with SS (Figure 1). Some autoantibodies commonly found in patients with SS, in particular anti-Ro and anti-La, are directed against ribonucleoproteins that are ubiquitously expressed. Interestingly, these autoantibodies may exert effects locally, at the exocrine gland level, where they affect cellular function and may contribute to acinar cell apoptosis.

ANTI-ACINAR AUTOIMMUNITY

The most distinctive feature of SS is the development of a well-organized immune response in exocrine glands. Which factors initiate this response and allow it to become chronic are unknown. Proposed mechanisms include viral infections and acinar cell apoptosis. Alternatively, products of an underlying systemic autoimmune response, for example anti-Ro antibodies, may initiate a local inflammatory response by inciting acinar cell damage that is amplified and perpetuated by the infiltrating immune cells.

Local lymphocyte infiltration

Chemokines are small molecules that exert powerful chemoattractant effects on a variety of cells. The local secretion of chemokines guides the amount and type of cells that infiltrate and therefore cause inflammation in a tissue. Therefore, the type and abundance of chemokines present in a tissue determine the intensity of inflammation and its characteristics. High concentrations of several chemokines including CCL5, CCL3, CCL17, CCL18, CCL19, CCL21, CXCL9, CXCL10, CXCL11, and CXCL13, have been reported in affected glands of patients with SS[7]. CXCL12 and CXCL13 are strongly associated with the development of ectopic germinal center (GC) like structures[8] and CXCL10 has been proposed as a biomarker for early salivary gland inflammation[9].

The therapeutic usefulness of targeting chemokines has been tested in animal models. In MRL/lpr mice, the use of an N-terminal-truncated IP-10 analogue that possessed IP-10 receptor antagonist capacities ameliorated the progression of autoimmune sialadenitis[10]. Likewise, CXCL13 blockade reduced the number of lymphocytic foci in salivary gland in the non-obese diabetic mouse model when administered before disease onset[11]. Conversely, CCL28 a mucosal chemokine that attracts CD4 and CD8 T cells was diminished in saliva of

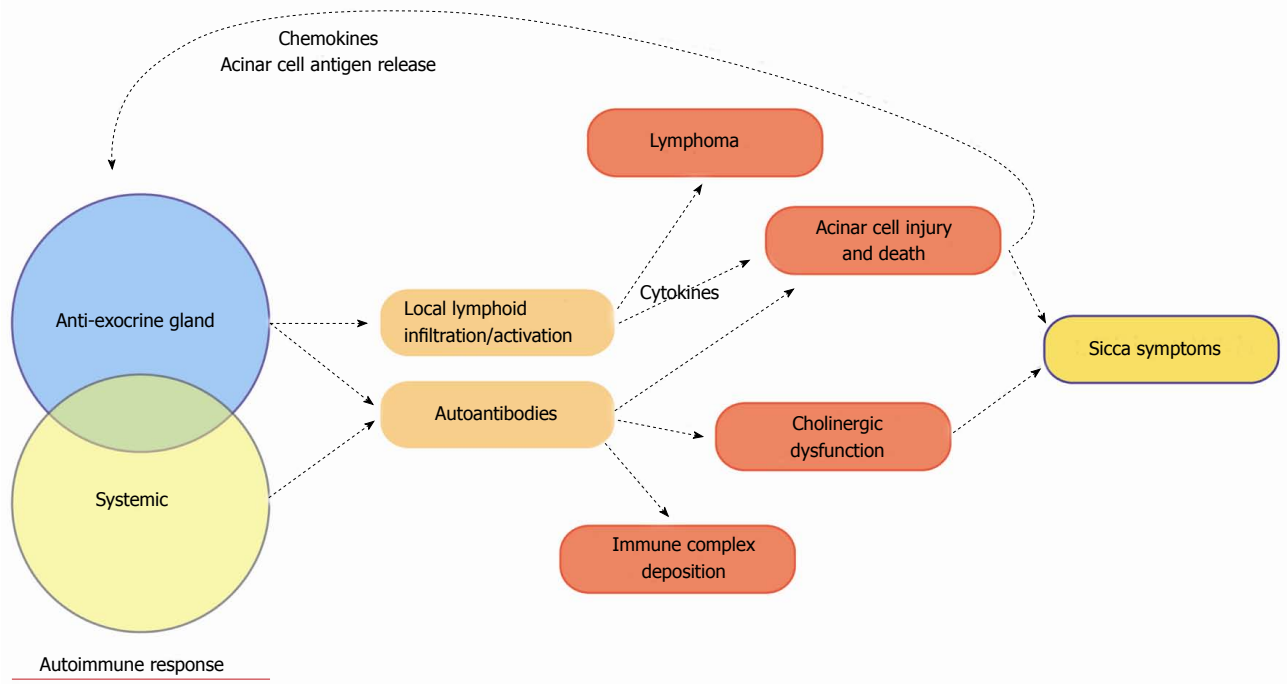

Figure 1 Patients with Sjögren's syndrome develop an autoimmune response that has systemic and local (anti-exocrine gland) components. Both responses are most obviously manifested by the presence of autoantibodies directed, respectively, against ubiquitous (*e.g.*, Ro) and tissue-specific (*e.g.*, muscarinic receptor 3) antigens. The anti-exocrine gland response is also notable by the presence of chronic inflammatory cellular infiltrates composed of activated lymphocytes. Autoantibodies contribute to systemic manifestations through immune complex deposition, but also contribute to sicca symptoms by affecting cholinergic function and causing acinar cell apoptosis. Cellular infiltrates directly affect exocrine gland function by inducing local inflammatory damage and local B cell proliferation probably precedes lymphoma development. Injury to acinar cells perpetuates the local immune response by releasing antigens and inflammatory mediators (*e.g.*, IL-1, type Ⅰ IFN). IL-1: Interleukin-1; IFN: Interferon.

patients with SS[12]. These preliminary data support the hypothesis that blocking chemokines may represent a therapeutic strategy in SS.

Mechanisms of glandular destruction

Cytokine gene knock-out mouse models as well as clinical evidence have shown that effector CD4 T cells with different functional differentiation programs (including Th1, Th2 and Th17) are involved at the initiation, perpetuation, and progression of the disease[13,14]. However data concerning the time when each of these responses is most relevant is conflicting. Some authors propose that a Th2 response dominates in early lesions[15] while others consider that Th1 and Th17 cytokines are essential for the induction and/or maintenance[7]. Th1 cells produce IFN-γ and IL-2 whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13[16,17]. On the other hand, Th17 cells represent a major source of IL-17A, IL-17F, and IL-22. Thus blocking one or a cluster of cytokines may be potentially useful therapeutic strategies (Figure 2).

Cytokines produced by innate immune cells and non-immune cells

Increased levels of IL-1 in saliva and peripheral blood of patients with PSS have been reported[18]. In the salivary and lacrimal glands, IL-1β production from infiltrating immune cells and also from local epithelial cells has been described[19]. This illustrates how damaged acinar cells may promote the initiation and perpetuation of the response that will further injure them by facilitating their apoptosis and contributing to the recruitment and local activation of T cells[19]. The IL-1R antagonist, anakinra, demonstrated therapeutic benefits as a topical treatment for dry eye in a spontaneous mouse model of autoimmune keratoconjunctivitis sicca that mimics SS[20]. On the other hand, a randomized, double-blind, placebo-controlled trial of IL-1 blockade did not find a significant reduction in fatigue in PSS[21].

Type Ⅰ interferons are produced by virtually every cell in response to the detection of intracellular infection. They act as danger signals that alert neighboring cells to the presence of pathogens and induce the local influx of inflammatory cells. Expression of genes that are induced by IFN is commonly used as a surrogate measure of IFN levels in a tissue. In salivary glands and in cells from the peripheral blood of patients with SS there is increased expression of IFN-driven genes[22,23]. High levels of type Ⅰ IFN may contribute to the pathophysiology of SS by mediating the recruitment, activation, and differentiation of inflammatory cells, by promoting the secretion of other cytokines such as B cell activating factor by monocytes, and by inducing the expression of pro-apoptotic molecules (*e.g.,* Fas and FasL)[24]. In the context of SS, high levels of type Ⅰ IFN are probably released locally as a result of cellular injury mediated by viruses and perhaps other non-infectious agents, and may also result from the detection of nucleic acid-containing immune complexes[25]. Acinar

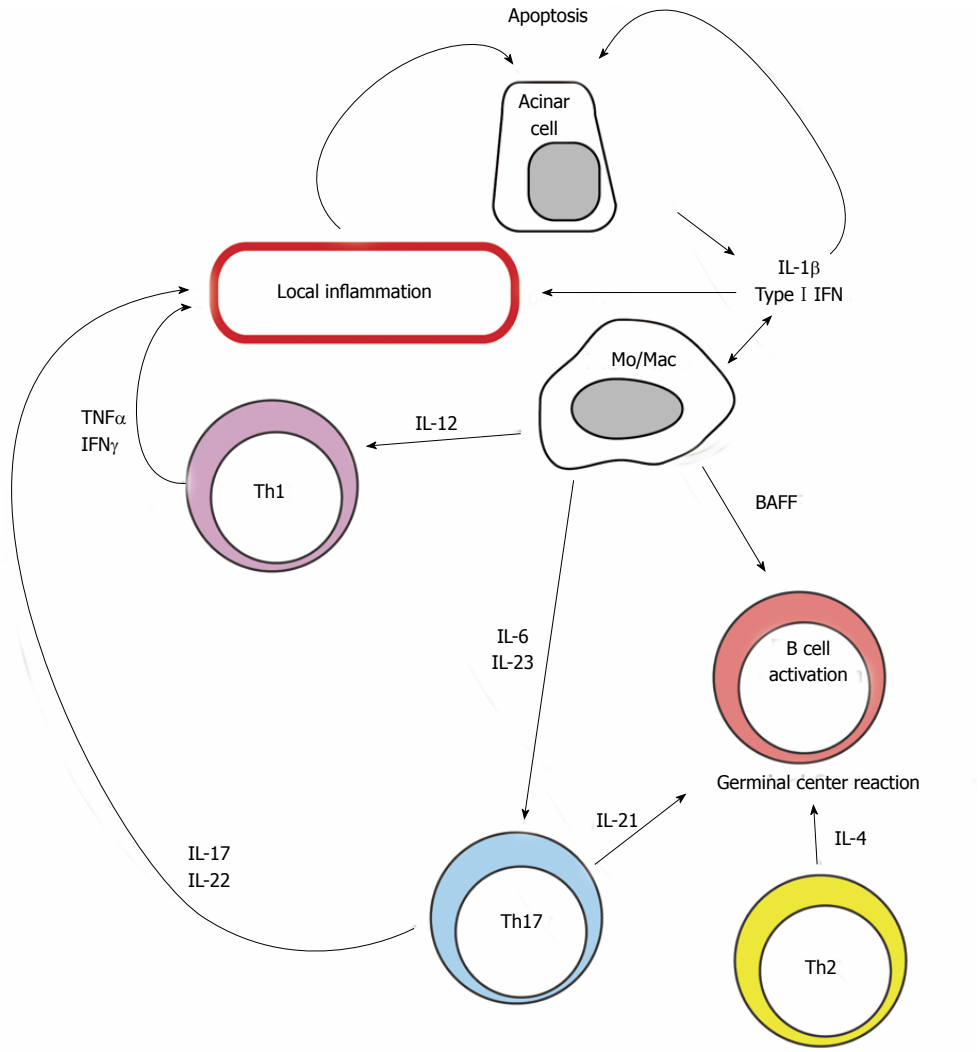

Figure 2 T cells and B cells are recruited into exocrine glands where they mount a chronic inflammatory response. Local production of IL-1β and type I interferons (IFN) by epithelial cells and innate immune cells contributes to local inflammation and promotes apoptosis of acinar cells. Clones of activated T cells with varied effector differentiation support local inflammation and contribute to acinar destruction. IL: Interleukin; BAFF: B cell activating factor.

production of these cytokines may promote local inflammation, whereas systemic production may facilitate the inflammatory response through their pro-inflammatory action in immune cells. For these reasons, therapeutic interventions targeting the type I IFN signaling pathway may be beneficial to patients with elevated type I IFN status. Currently two anti-IFN-α monoclonal antibodies, sifalimumab and rontalizumab are being tested in systemic lupus erythematosus (SLE) patients, but there are still no trials in SS[26].

Th1 response

CD4 T cells that are primed in the presence of IL-12 become Th1 cells. They promote inflammation through the production of IFN-γ and TNF-α, powerful cytokines that affect the behavior of macrophages, T cells, and B cells[27]. Early work reported that T cell differentiation in patients with SS was skewed towards a Th1 response because serum levels of IFN-γ were high and Th1 cells were more abundant in the peripheral blood of patients with SS than in healthy controls[28,29].

The pathogenic capacity of IFN-γ is further supported by work in NOD mice where genetic deletion of this cytokine or its receptor prevented the development of an anti-salivary gland autoimmune response. Of note, IFN-γ deletion was also associated with decreased apoptosis of acinar cells, suggesting that Th1 cells might also damage salivary glands through this mechanism[30].

Th17 response

Th17 cells represent potent pro-inflammatory CD4 T cells that promote the infiltration of innate and adaptive immune cells into tissues by producing cytokines (*i.e.,* IL-17A, IL-17F, IL-22) that act mainly on epithelial and endothelial cells by inducing the production of chemotactic molecules[31]. Th17 cells are generated when naïve CD4 T cells are primed in the presence of TGF-β and pro-inflammatory cytokines such as IL-1, IL-6, and IL-21, and have been reported to be involved in the pathogenesis of several autoimmune diseases, including SLE[32]. IL-17 and other Th17-related cytokines have been found in high levels in the saliva and in

salivary gland tissue of patients and mice with SS[33,34], suggesting the presence of a Th17-type inflammatory response.

Retinoic acid-related orphan receptor gamma (ROR-γ) is a transcription factor that is necessary for the development and function of Th17 cells[35]. Transgenic overexpression of ROR-γ led to the development of a severe spontaneous sialadenitis driven by the infiltration of salivary glands by CD4 T cells[36]. Interestingly, salivary gland-infiltrating CD4 T cells in ROR-γ transgenic mice produced not only IL-17, but also IFN-γ, IL-4, IL-21, and other cytokines. In fact, IL-17 was not necessary for disease development since genetic deletion of *IL17a* did not modify the frequency or pathology of the disease[36].

IL-6 is increased in tears, saliva and serum of PSS patients[37]. This cytokine has been associated with B cell hyperactivity and promotes STAT-3 activation. In Sle1.Yaa mice that develop a lupus- and Sjögren's-like syndrome, deficiency of IL-6 ameliorated the autoantibody production and salivary gland inflammation[38]. Currently IL-6 blocking therapy (tocilizumab) is being evaluated in a phase Ⅲ randomized controlled trial in PSS[39].

IL-23, which stabilizes the phenotype of Th17 cells, is also significantly increased at both the protein and the mRNA levels in the salivary glands of patients with PSS[40,41]. Clinical trials using anti-IL-23 (ustekinumab, briakinumab, tildrakizumab and guselkumab) have been performed in psoriasis, yet there is still no information in PSS[42].

The importance of IL-17 in the development of SS has been shown in mouse models. Inhibition of IL-17 activity by expression of a soluble IL-17R:Fc fusion protein restrained the development of SS in C57BL/6.NOD-Aec1Aec2 mice[43]. The use of anti-IL-17 monoclonal antibodies (ixekizumab and secukinumab) or its receptor IL-17RA (brodalumab) are being evaluated in rheumatoid arthritis and psoriatic arthritis, but trials in SS using these treatments are lacking[42]. However the use of anti-IL-6 receptor (tocilizumab)[44] or a fusion protein of the extracellular domain of CTLA-4 and human IgG1 (abatacept) significantly decreased the abundance of Th17 cells in the peripheral blood of patients with PSS[45].

IL-22 is produced by Th17 and NK cells. IL-22 is over-expressed in the serum and salivary glands of patients with PSS and correlates with hyposalivation, the presence of autoantibodies and the focus score[46]. The IL-22 axis seems to be functionally dependent on IL-18 signaling and both IL-18 and IL-22/IL-22R1 are over-expressed in PSS patients with non-Hodgkin lymphoma[47]. The potential role of blocking IL-22 in SS pathogenesis remains to be evaluated in mouse models and humans.

IL-21 has pleiotropic effects promotes the differentiation, proliferation, and survival of B and T cells. IL-21 exerts pro-inflammatory functions by inducing the expression of IL-6 and ROR-γ and thus contributing to the generation of Th17 cells and attenuating Treg induction. Patients with PSS have elevated serum IL-21 levels and expression of IL-21 at lymphocytic foci and periductal areas of minor salivary gland biopsies has been documented[48]. Moreover, a genetic polymorphism located upstream of IL-21 (IL2-IL21 intergenic region) was associated with PSS in a Latin-American population[49]. The suppression of local IL-21 expression at the submandibular glands using an shRNA-encoding lentivirus reduced the lymphocyte infiltration and improved salivary gland function in NOD mice[50]. Nowadays there is only a phase Ⅰ study of a monoclonal anti-IL-21 in humans, so further research is needed[51].

Th2 response

Th2 cytokines are also produced in the glands of patients and mice with SS. Evidence indicates that contrary to Th1 and Th17 cells that instigate local inflammation, Th2 cells participate in the pathogenesis of SS by facilitating the autoantibody response.

IL-4 is present in minor salivary gland biopsies of patients with PSS[7]. In mouse models, it participates in the IgM to IgG1 isotype switch *via* the JAK/STAT6 signal transduction pathway. NOD mice deficient in IL-4 or in STAT6 still develop focal salivary gland infiltrates, but they fail to produce anti-muscarinic acetylcholine type-3 receptor antibodies of IgG1 isotype and therefore exocrine gland dysfunction is avoided[52,53]. A salivary proteomic biomarker profile study found IL-4 as part of a 4-plex and 6-plex biomarker signatures in PSS[54]. A study reported a similar distribution of IL-4RA genetic variants among PSS and healthy controls. However, the haplotype ARSPRV was significantly more frequent among patients with parotid gland enlargement and positive immunological parameters, suggesting that differential response to IL-4 in epithelial tissue might affect disease phenotype[55]. The potential role of blocking IL-4 in PSS remains to be evaluated.

IL-13 mRNA is present in salivary gland of patients with PSS[15], and SS patients with antiSSA/Ro antibodies had significantly higher IL-13 levels than patients without this autoantibody[56]. Blockade of IL-13 activity in the ID3-KO mice model improved salivary gland function[57]. Recently anrukinzumab, a humanized anti-IL-13 antibody, was evaluated in ulcerative colitis[58], however there are not trials in PSS patients.

IL-10 mRNA expression is increased in salivary glands of PSS patients[59]. In a transgenic murine model, IL-10 induced lymphocytic infiltration and apoptosis of glandular cells[60]. The use of anti–IL-10 mAb administration has been only explored in a small open study of lupus patients with improvement of disease activity[61]. Conversely, IL-10 has also been considered as an anti-inflammatory agent. For instance, IL-10 is produced by regulatory B cells that are increased in clinical inactive PSS patients[62]. Thus future research to elucidate the complex function of IL-10 is needed.

Several cytokines contribute to the pathogenesis of SS (*e.g.,* IFN-γ, IL-4, IL-6, IL-21) signal through Janus kinases (JAKs) and signal transducer and acti-

vator of transcription (STATs) transcription factors. The activity of the JAK-STAT pathway is negatively regulated by suppressors of cytokine signaling proteins. JAK/STAT kinase inhibitors suppress the inflammatory cytokine function and stimulate the production of anti-inflammatory cytokines such as IL-10[63]. A pan-Janus kinase inhibitor with a higher affinity for JAK1 and 3 (tofacitinib) is currently available and has been used successfully in patients with rheumatoid arthritis[64].

Induction of apoptosis

Apoptosis of epithelial cells from exocrine glands represents an important phenomenon in the pathogenesis of SS. The increased rate of acinar cell death has direct consequences on the production of saliva and tears, the most frequent clinical complaint of patients with SS. Moreover, it may represent a key local factor responsible for the recruitment of inflammatory cells that amplify the damage to the glandular tissue.

Although most studies have found abnormally high levels of apoptosis in ductal and acinar epithelial cells in salivary glands of patients with primary SS[65], it has been difficult to determine whether increased local apoptosis and release of glandular antigens initiates the local autoimmune response in susceptible patients, or whether self-reactive T cells infiltrate the glands guided by their antigen specificity and cause apoptosis in susceptible cells (*e.g.,* cells that express FasL)[66].

Epithelial cells from salivary glands of patients with SS are susceptible to apoptosis. Because they express high levels of both Fas and FasL, it has been proposed that apoptosis may be triggered in them in an autocrine or paracrine manner as well as in response to glandular infiltration of Fas-bearing T cells[65].

The IgG fraction of sera from patients with SS has the capacity to penetrate living A-253 cells (a human salivary gland cell line) and trigger caspase activation and apoptosis, raising the possibility that autoantibodies produced in SS may contribute to the pathogenesis of the disease by inducing death of epithelial cells[67].

Autoantibodies

B cell hyperactivity and the presence of high titers of autoantibodies are common phenomena in patients with SS. Germinal center formation is observed in the glandular tissue of up to 25% of the patients with SS, usually in patients with severe disease[68]. Not surprisingly, patients with germinal centers have higher titers of autoantibodies (*e.g.,* rheumatoid factor, anti-Ro) and a higher risk for development of lymphoma[68].

How local B cell activation and proliferation contributes to the pathogenesis of SS is not well understood[69]. B cells may produce cytokines, including IL-6 and IL-10 that affect the activation and function of infiltrating immune cells. Also, they may act as antigen presenting cells, thereby amplifying the autoimmune response. Finally, autoantibodies may contribute to acinar cell dysfunction and death by affecting cholinergic signaling and by inducing apoptosis[67].

CONCLUSION

Patients with SS develop an autoimmune response that has systemic and local components. The clinical manifestations of the disease are the consequence of pathological processes that result from both autoimmune responses. Exocrine gland infiltration and the development of a local chronic inflammatory response is probably primarily responsible for the destruction of the acinar cells and consequently for the development of sicca symptoms. This immune response is complex and comprised of activated T cells and B cells and cannot be attributed to a single cell type and a unique differentiation program. A more thorough understanding of disease pathogenesis, including information obtained in animal models, will allow us to better understand which therapies may be more useful and may stop the destruction of the glandular tissue that leads to the irreversible changes that affect most patients with SS.

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MINIREVIEWS

Effects of exercise on antibody production

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Abstract

In this review, we have focused on the effects of exercise on infection or antibody production. In the past, exercise immunologists largely focused on exercise and its effects on infection. Research on the effects of exercise on antibody response began in the 1970s with a primary focus on whether regular exercise helps to minimize the risk of infection. Positive results from these early studies indicated that exercise affects higher survival rate. Based on the results of these studies, researchers then investigated the exercise-induced elevation of plasma antibody levels. It has been suggested that exercise of moderate intensity could be a helpful and effective adjuvant for human health. Other studies have examined the effects of exercise on antibody-producing cells, and the levels of protection conferred by the produced antibodies. We have attempted to summarize the current understanding of exercise-induced elevations in plasma antibody levels. We also propose some future directions for investigating the relationship between exercise and antibody response.

Key words: Antibody; Exercise; Circulating IgG; Liver; β2-microglobulin

Core tip: Exercise-induced plasma antibody elevation is now recognized as a key recall response to vaccine antigens. In exercised mice, antigen-specific antibody producing cells harvested more and their elongated half-life of the produced antibodies, resulted in their evoked secondary IgG responses. Here, we review the effects of exercise on antibody response.

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INTRODUCTION

The relationship between exercise and infection has long interested exercise immunologists; however, it is not yet fully understood[1]. Immune reactions to pathogens are generally evaluated by the relative level of plasma antibodies. The production of antibodies is a major protective response against infections. In this review,

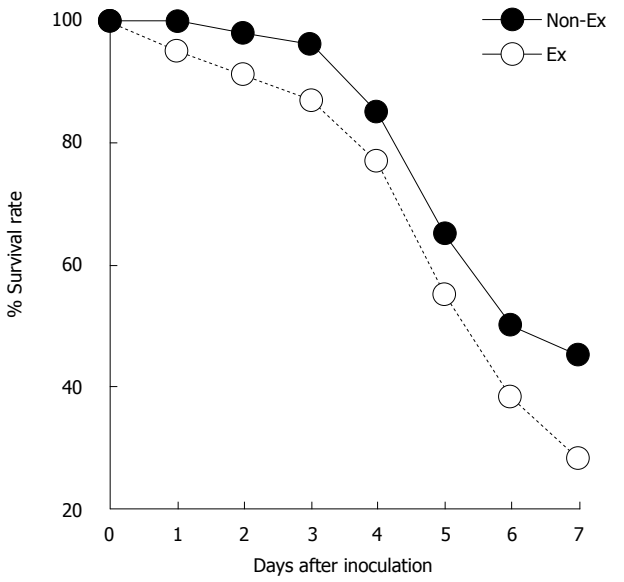

Figure 1 Exercise enhances survival rate in mice infected with *Salmonella typhimurium*. Mice voluntarily trained on exercise wheels for 16-18 d (EX) and were infected with *Salmonella typhimurium*. These EX mice exhibited a small but significant increase in survival rate (34/77) compared with sedentary control (Non-EX) mice (23/79) after 7 d (Modified from Cannon *et al*[3]). EX: Exercise; Non-EX: Non-exercise.

we have provided the historical background of immune function research, and its relationship to exercise. We then outline the current state of knowledge regarding the effects of exercise on antibody-producing cells and extend antibody half-life. We have also discussed potential mechanisms by which moderate levels of exercise can prevent infections.

EXERCISES ENHANCE SURVIVAL IN ANIMALS INFECTED WITH PATHOGENIC MICROORGANISMS

Regular and consistent exercise is known to confer substantial benefits to the immune system, and prevent infection over the long term[2]. Cannon *et al*[3] were the first to report the effects of exercise on infection. In their study, mice were infected with *Salmonella typhimurium*; mice that had partaken in some form of exercise had a significantly higher survival rate than sedentary mice. This initial study demonstrated the beneficial effects of exercise on the immune system during an infection (Figure 1). Stressful exercise can exacerbate infections[4,5], while moderate levels of exercise can attenuate disease severity[4,6]. Lowder *et al*[6] showed that exercise of moderate intensity protected mice from death against the influenza virus. In this study, mice were administered influenza virus A/PR/8/34, a virulent H1N1 strain[7]. Mice were then made to exercise at a moderate intensity (8-12 m/min for 20-30 min over four consecutive days) or over a prolonged period (8-12 m/min for 2.5 h over 4 consecutive days), with some mice used as sedentary controls. Results from a majority of studies support the view that exercise confers a higher survival rate to an infection[6,8]. A limitation of these animal studies is that they have examined primary responses, but not secondary responses to infection. They have been repeatedly confirmed in many studies. First, mice that are made to exercise show significantly higher survival rates than sedentary mice following the injection of an antigen. Second, continuous exercise leads to significantly higher survival rates for active mice than in sedentary mice. Third, antigen-specific antibody responses are more enhanced in active mice than in sedentary mice.

EXERCISE INDUCES ANTIBODY PRODUCTION

Following exercise and infection studies, exercise immunologists focused on exercise-induced plasma antibody elevation. The antibody response to antigens is a complex process; however, it is important to understand this if the potential for exercise to alter the response is to be investigated. Antibody responses are T cell-dependent and comprise of antigens that are soluble proteins. The first step in the response to an antigen is its uptake and presentation. This is usually performed by specialized antigen presenting cells (APCs), such as macrophages, dendritic cells, or B cells. During T cell-dependent responses, naive B cells recognize the presented antigen, and mature after cognate interaction with T helper (Th) cells. However, for T cell-independent responses, B cells do not need to interact with APCs or T cells. Mature B cells, including plasma cells, are able to secrete antibodies and have a "memory" of encountered antigens.

A significant amount of research has been conducted to investigate whether moderate exercise induces antibody production[1,9-13]. In most of these studies, the administered antigen was not infectious. Liu *et al*[10] tested the hypothesis that exercise induces the production of plasma antibodies. They examined the plasma antibody levels of sedentary and active (running for 10 min, twice a day) mice following infection with *Salmonella typhi*. Antibody titers in the active mice were significantly higher (2.76-fold) than those in the sedentary mice over the entire experimental period. The antibody titers in the active mice were 2.76-fold higher than those in sedentary mice. Liu *et al*[10] observed a primary antibody response after initial exposure to an antigen. Subsequent exposure to the same antigen led to a strong secondary antibody response, resulting in long-lasting immunity. The effects of exercise on secondary antibody responses have been tested in young mice[1,9] and rats[11].

A number of investigations into the influence of exercise on antibody response have been conducted, largely focusing on the secondary antibody response (Figure 2). Exercise immunologists were interested in the dramatic changes to the secondary antibody

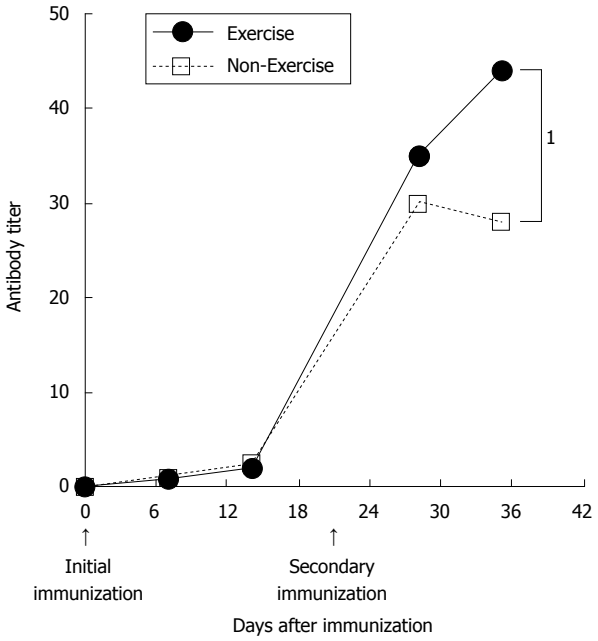

Figure 2 Effects of physical training on the murine immunological response. Serum IgG levels in active (exercise) and control (non-exercise) mice. Arrows indicate the time of immunization. [1]Significant difference between Exercise and Non-Exercise groups (Modified from Douglass[1]).

response in active mice (Figure 2). Moderate exercise, such as voluntary wheel running[12] or treadmill running (8-15 m/min[13] has been shown to have a pronounced effect on the secondary antibody response. These findings indicate that is valuable information encouraged exercise immunologists to investigate other additional components affecting the immune response. Thus, since an early era, investigations of exercise effects on immunity, which reflect the secondary antibody response during exercise. Moderate exercise could be an adjuvant to vaccination, and further research into this is required.

EXERCISE INDUCES THE SECONDARY ANTIBODY RESPONSE

Exercise is known to affect the secondary antibody response in animal models. In mice, the primary IgG response to tetanus toxoid (TT) was not enhanced by moderate exercise; however, in active mice there was an increased IgG response following a booster dose[12]. Subsequent research findings revealed that booster shots resulted in enhanced antibody production in mice that exercised[10-13]. Antibody responses to TT require recognition of the antigen by Th cells and cooperation with antigen-specific B cells and T lymphocytes. The interaction between Th and B cells involves antigen presentation by B cells to differentiated T cells, activation of Th cells, and the expression of membrane and secreted molecules by the Th cells that bind to and activate B cells. It remains unclear why moderate exercise impacts upon the secondary antibody response, but not the primary response.

Factors involved in the increased secondary antibody response in mice have been investigated in detail by Suzuki *et al*[12]. They investigated the effects of exercise on the number of cells producing specific IgG in mice by using ELISPOT assays[14]; secondary antibody responses to TT were significantly higher in the active group than in the sedentary group. Exercise induce secondary antibody response to antigen is valuable information and encouraged exercise immunologists. Thus, since an early era, investigations of exercise effects on immunity were beginning.

ROLE OF ENDOGENOUS OPIOIDS IN THE EXERCISE-INDUCED SECONDARY ANTIBODY RESPONSE

The mechanism(s) behind increased specific secondary antibody responses in moderately active animals is unclear[12]. It has been suggested that endogenous opioids are involved in these enhanced secondary antibody response. Enkephalins were first observed in the brain and the endocrine system. Both endorphins and enkephalins are important regulators of pain[15,16]. Recently, inflammatory cells were shown to produce and release these opiates. Endorphins appear to be involved in immune functions[17,18], pain modulation[19], and the exercise pressor response[20-22]. The role(s) of these endogenous opiates with respect to the exercise-related processes they modulate require clarification, in particular at the cellular level.

Kapasi *et al*[13] immunized mice with an antigen and treated them with a placebo or an opioid antagonist (naltrexone); control mice received no intervention. Mice were further subdivided into sedentary and active groups, with those in the active group made to exercise for 8 wk and following a booster shot. During the secondary response, high antibody titers were seen in the immunized active mice. However, the antibody titer was not increased in naltrexone-treated mice. It has also been concluded that norepinephrine appears to mediate suppression and stimulation of antibody synthesis. Enhancement of the antibody response by endogenous opioids is dependent on the dose of intravenous injections[23]. Antibody production occurs as a result of interactions between the retained antigen on follicular dendritic cells, B lymphocytes, and Th lymphocytes[24].

Exercise induced secondary antibody production mechanism most likely involves opioids binding to specific receptors on B and T cells[25]. Endogenous opioids influence the antibody response through receptors on Th ($CD4^+$) cells and by stimulating proliferation[26]. This is likely a result of IL-4 production being induced, with IL-4 increasing splenic B cell survival[27]. Further research is required to determine whether the effects of exercise on specific enhanced secondary antibody responses are due to such a mechanism.

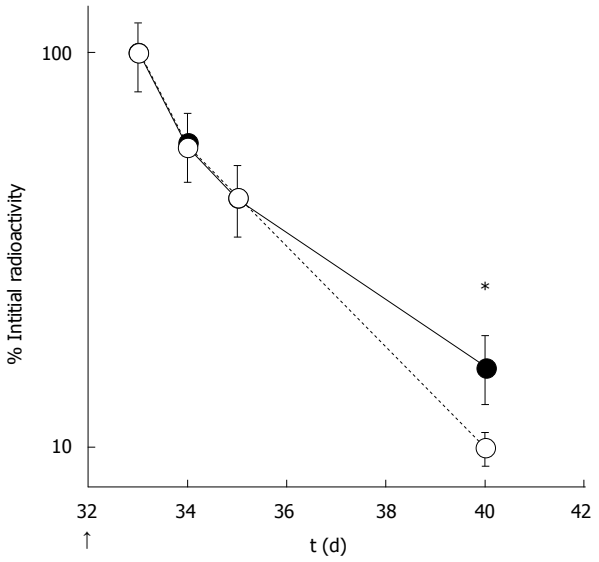

Figure 3 Clearance of radiolabeled IgGs from active (black circles) and sedentary (white circles) mice (Modified from Suzuki *et al*[12]). *Significant difference between active and sedentary mice.

EFFECTS OF EXERCISE ON IGG HALF-LIFE

The mechanism by which exercise induces elevation of secondary IgG levels is related to IgG half-life[12]. In several species, it was found that the clearance rate of IgG is highly dependent on its concentration in plasma[28]. A physiological concentration of IgG half-life is approximately 10 times longer than at high concentrations[28]. A significant fraction of IgG proteins undergoes endocytosis[29]. IgG does not localize to the lysosome, but is instead redirected to the cell surface and released into plasma or interstitial fluids. The recycling of IgG is mediated by the Brambell receptor, FcRn, which binds to IgG in a pH-dependent manner[30,31]. Within the acidified environment of the early endosome, IgG binds strongly to FcRn. IgG-FcRn complexes are not delivered to the lysosome for catabolism[32]; instead, they migrate to the cell surface for fusion with the cell membrane. The FcRn receptor shows virtually no affinity for IgG at a physiological pH. Upon fusion of the sorting vesicle with the cell membrane, IgG dissociates from the receptor and is rapidly released into the extracellular fluid. The clearance of IgG is increased by approximately 10-fold[31], which is consistent with a recycling efficiency of 90% in wild-type animals expressing FcRn.

A series of studies examining the effects of exercise on IgG clearance have been published[12,33]. Exercise induced secondary antibody response to antigen was reported by Suzuki *et al*[33]. They investigated factors that lowered non-specific ^{125}I-IgG clearance from plasma after a booster immunization (Figure 3). A high concentration of circulating antibody can result in lower clearance of antibodies from circulation. The reasons underlying the low clearance of ^{125}I-IgG from circulation in active mice remain to be resolved. The IgG homeostatic mechanism is dependent on the Fc region of IgG[34]. A possible downstream mediator that could explain the lower level clearance is the FcRn receptor. FcRn is normally expressed in the vascular endothelium in the adult rodent, where it is thought to confer a protective function[35]. A functional FcRn molecule is dependent on its dimerization with β2-microglobulin (β2m)[36]. There is strong evidence to indicate that β2m-deficient mice have an abnormally short IgG half-life and reduced homeostatic IgG levels[37]. Suzuki *et al*[33] reported on the effects of β2m expression in the liver, which were implicated in protecting against IgG catabolism. Mice were intraperitoneally immunized with TT to induce primary and secondary antibody responses. The authors found that the half-lives of TT-specific IgG were prolonged in active mice, with significantly higher blood IgG concentrations in active mice compared with those in sedentary mice. In the active mice, radiolabeled IgG concentrations in the liver were higher than those in sedentary mice, and this was confirmed by immunohistochemical analysis. Expression of the *β2m* gene was upregulated in the livers of active mice. There was a significant correlation between the accumulation of radiolabeled IgG in the liver and its concentration in the blood. In addition, there was a significant correlation between extracted total hepatic IgG and β2m in the liver.

EFFECTS OF EXERCISE ON THE SECONDARY ANTIBODY RESPONSE IN AGED ANIMAL MODEL

Aging is a natural process and is associated with a decline in the normal functioning of the immune system that can be described by the term "immunosenescence"[38]. Accelerated degradation of host immune responses is a key sign of immunosenescence, and can lead to the onset of opportunistic infections[39]. Immunosenescence can also result in poor vaccine responses[40] and the increased incidence of infection that is often seen in the elderly. Restoration of immunological function is expected to have a beneficial effect in reducing pathology and maintaining the health of older individuals. Moderate exercise has been used as an intervention to counteract the aging immune system. Regular exercise has been associated with enhanced vaccination responses[41,42]. Aging is associated with declines in humoral and cellular immunity[43], and therefore reduced immune function. The age-related decline in the function of major cells that take part in the antibody response are reflected by the secondary antibody response[44]. Kapasi *et al*[45] focused on age-related changes in immune function and the effects of exercise, and their study clearly showed that older mice exhibited a secondary antibody response similar to that seen in young control mice after exercise. Thus, intense exercise exerts positive effects on the secondary antibody response in old animals.

EFFECTS OF EXERCISE ON THE ANTIBODY RESPONSE IN HUMANS

A beneficial effect of exercise on the secondary antibody

response to infectious diseases is improved. The use of exercise to augment vaccine responses in humans has been explored, with positive results observed[41,46-49]. Moderate aerobic exercise in older adults[48] and muscle-damaging eccentric contractions in younger adults[50] have been shown to increase immune responses to influenza vaccination. In addition, several cross-sectional studies have found that physically fit[51] or active elderly individuals[52] exhibit elevated antibody responses to recall vaccinations. Shuler *et al*[46] examined antibody titers in response to influenza vaccination in college students. Measures of physical fitness and physical activity were taken, but neither was found to be associated with the magnitude of the antibody response.

In contrast, several cross-sectional studies of older adult populations have all reported enhanced antibody responses to vaccinations in participants with high levels of physical fitness[51], or who were physically active[46,52,53]. This contrast in effects of chronic exercise on vaccination responses elicited in older adult populations, but not in younger populations, was exemplified by Smith *et al*[53]. They compared the immune response to a novel antigen, keyhole limpet hemocyanin (KLH). The authors showed that older active men demonstrated stronger antibody and cell-mediated responses to KLH than those in sedentary older men. The responses to KLH in younger men were similar, regardless of their activity habits. Woods *et al*[54] demonstrated that a 10-mo regimen of cardiovascular exercise (60%-70% maximal oxygen uptake, 45-60 min, three times a week) in previously sedentary older adults resulted in increased seroprotective maintenance, compared with participants who took part in flexibility training over the same period. An increased response to novel antigens has also been observed following chronic exercise. After KLH vaccination, IgG1 and IgM concentrations were greater in participants who had completed a 10-mo cardiovascular training program (11% increase in VO_2 max) than in control participants (1% increase in VO_2 max)[55]. The majority of results from previous studies supports the hypothesis that regular exercise improves immune function. This is reflected in enhanced antibody or cell-mediated responses to vaccination, especially in older adults. In general, for the majority of papers we reviewed, vaccine response was assessed *via* antibody titer, the humoral component of the immune response. Previously published findings support the hypothesis that exercise of a moderate intensity appears to enhance immune responses in animals. Therefore, the development of effective exercise regimens that promote antibody responses to vaccination would assist in preventing and reducing the risks of infection.

CONCLUSION

In this review, we have highlighted how moderate exercise interventions can improve immune responses to antigens or pathogens. Currently available evidence shows that exercise has important modulatory effects on antibody responses, and possibly on immune functions. These effects are mediated by a diverse range of factors, including the functions of antibody producing cells, endogenous opioids, aging, and IgG half-life. Most exercise studies have focused on antibody production, with more work required in this area. Almost all studies have investigated the effects of moderate exercise on immune function; however, it remains unknown if exercise is capable of modulating specific antibody-producing cells. While exercise might help boost adaptive immunity by increasing the thymic output of naive T cells[56], and/or by purging senescent T cells[57], further research is required to confirm this. This would provide us with insight into the types of vaccination, which can be ameliorated with exercise. As molecular biological techniques are incorporated into exercise immunology studies, a greater understanding of the pathways of cell activation and regulation should be forthcoming.

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