Journal of Immunology Research

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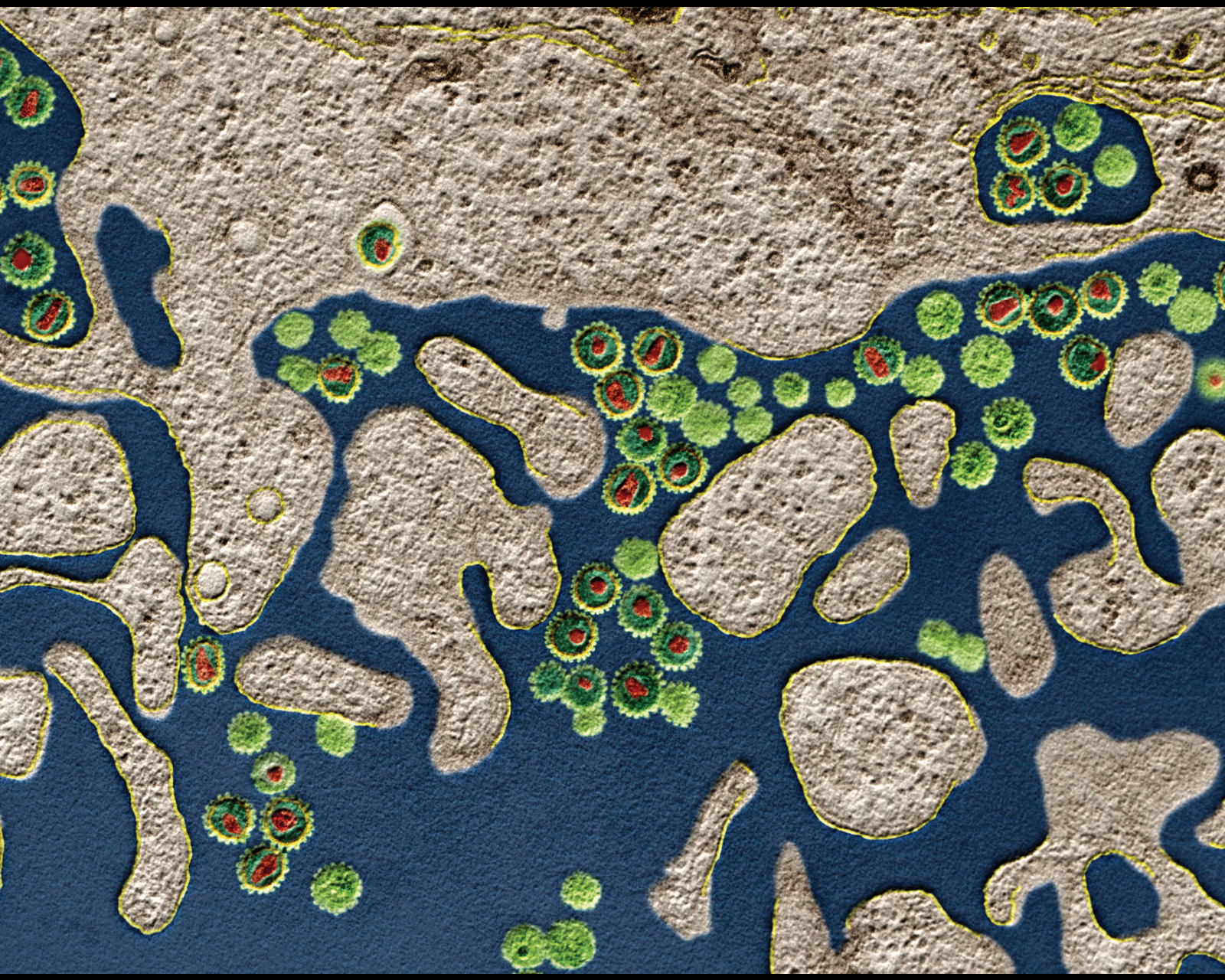
Guest Editors: Jong-Young Kwak, Mizuko Mamura, Jana Barlic-Dicen, and Evelin Grage-Griebenow



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Editorial Board

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 615130, 2 pages
http://dx.doi.org/10.1155/2014/615130

Editorial

Pathophysiological Roles of Cytokine-Chemokine Immune Network

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Received 5 August 2014; Accepted 5 August 2014; Published 11 September 2014

Cytokines are small proteins or glycoproteins, and chemokines form a group of smaller cytokines with chemotactic properties that are produced by a variety of cells. Cytokines and chemokines exert crucial roles in the development, homeostasis, activation, differentiation, regulation, and functions of innate and adaptive immunity. Excessive and/or inappropriate production and actions of cytokines and chemokines are involved in the pathogenesis of infection, inflammation, allergy, autoimmune diseases, and immune-related diseases, such as diabetes, atherosclerosis, rheumatic arthritis, and cancer. Understanding the underlying mechanisms by which cytokines and chemokines exert their functions will lead to development of therapeutics for immune-related diseases. In this special issue, the articles and reviews discuss recent findings that highlight the pathophysiological role of cytokine and chemokines.

Effector and memory T cells are phenotypically and functionally heterogeneous. Upon antigenic stimulation, naïve $CD4^+$ T cells become effector Th1, Th2, or Th17 cells or regulatory T cells (Tregs). Effector and regulatory T cell subsets are generated in the presence of specific cytokines such as IL-12 and TGF-β, respectively. Transcription factors initiate and stabilize commitment toward the Th1 or Th2 lineage. T-bet (the T-box protein expressed in T cells) is the master regulator of Th1 differentiation [1]. A review article by S. Oh and E. S. Hwang summarizes the current state of knowledge regarding the molecular mechanisms that underlie the multiple roles played by T-bet in T helper cell development and fine-modulation of IL-2 production in Th1 cells. Interestingly, IFN-γ stimulation of dendritic cells (DCs) might have an equally important role in generating different effector T cells [2]. In this special issue, A. Visperas et al. describe several cytokines that are able to influence the generation of effector T cells by directly affecting T cells as well as targeting non-T cells. Inflammatory cytokine signaling also plays an important role in the pathogenic conversion of natural Tregs. The article by R. Takahashi and A. Yoshimura is a review in which the possibility is proposed that suppressor of cytokine signaling 1 (SOCS1) may protect Tregs from harmful effects of inflammatory cytokines, and SOCS1 upregulation maintains Treg functions. SOCS1 may be important in the pathogenesis of systemic lupus erythematosus through Treg plasticity, because SOCS1-deficient T cells induce lupus-like autoimmunity [3].

Both tumor-antagonizing and tumor-promoting inflammatory cells can be found in most neoplastic lesions. Inflammation can increase the risk of cancer by providing bioactive molecules from cells that infiltrate the tumor microenvironment, including cytokines and chemokines [4]. G. Landskron et al. review the roles of several inflammatory mediators,

including TNF-α, IL-6, TGF-β, and IL-10, in events of carcinogenesis, such as their capacity to generate reactive oxygen and nitrogen species; their potential mutagenic effect; and involvement in mechanisms for epithelial mesenchymal transition, angiogenesis, and metastasis. They also provide an in-depth analysis of the participation of cytokines in cancer that is attributable to chronic inflammatory diseases, such as colitis-associated colorectal cancer and cholangiocarcinoma.

Recent studies have shown that the circadian clock is responsible for the temporal dynamics of the immune system [5]. For example, joint stiffness and secretion of inflammatory cytokines in rheumatoid arthritis (RA) patients are influenced by the diurnal rhythm and peaks in the morning. RA is a devastating autoimmune disease that is characterized by progressive bone destruction and it was found that the circadian clock not only impacts arthritic symptoms but is also involved in the pathogenesis of RA [6]. Proinflammatory cytokines, such as IL-1, IL-6, IL-8, IL-11, IL-17, and TNF-α, are known to be osteoclastogenic [7]. In this special issue, S. M. Jung et al. discuss the osteoclastogenic role of the proinflammatory cytokines and immune cells in the pathophysiology of RA. Moreover, a review by A. Nakao summarizes recent advances regarding the emerging role of the circadian clock as a novel regulator of cytokines. K. Yoshida et al. describe the link between the circadian clock and inflammation, focusing on the interactions of various clock genes with the immune-pathways underlying the pathology of RA. In another article, Y. Nakamura et al. demonstrate that mechanical disruption of the suprachiasmatic nucleus (SCN) of the hypothalamus resulted in the absence of time of day-dependent variation in anaphylactic reaction in mice. These articles provide evidences that daily variations in cytokine levels are related to the pathophysiology of immune diseases.

Obesity, insulin resistance, and atherosclerosis are chronic inflammatory processes that are affected by the activation of innate and adaptive immunity [8, 9]. With regard to the role of inflammation of adipose tissue in obesity and obesity-related diseases, aberrant production of adipokines, cytokines, and chemokines in adipose tissue leads to inflammation in the tissue. A review article on this topic by L. Yao et al. covers the chemokine system and signaling in the development of obesity, insulin resistance, and plaque formation.

A clinical study by K. Theodoraki et al. demonstrates that IL-10 levels were elevated in patients that were subjected to major abdominal surgery procedures with a more liberal red blood cell transfusion strategy. Their results indicate that IL-10 may be an important factor in transfusion-associated immunomodulation. Expression of proinflammatory cytokines and chemokines has also been reported in patients with chronic venous insufficiency, such as varicose veins [10]. V. Tisato et al. demonstrate that EGF, PDGF, and RANTES were increased in varicose veins compared with general circulation, and a patient who exhibited recurrence of the disease 6 months after surgery showed higher levels of these factors compared with nonrecurrent patients. Therefore, V. Tisato et al. suggest that EGF, PDGF, and RANTES can be used as sensitive biomarkers of chronic venous insufficiency. Finally, J. Sturgill et al. examine the correlation of cytokine production in plasma of patients with fibromyalgia and they suggest that suppression of Th2 cytokines is related to immune dysregulation in patients with fibromyalgia.

Acknowledgments

We wish to thank the authors and reviewers for their contributions to this special issue.

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Mizuko Mamura
Jana Barlic-Dicen
Evelin Grage-Griebenow

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 263625, 15 pages
http://dx.doi.org/10.1155/2014/263625

Review Article

Cytokine-Mediated Bone Destruction in Rheumatoid Arthritis

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Received 24 March 2014; Accepted 27 May 2014; Published 10 September 2014

Academic Editor: Mizuko Mamura

Bone homeostasis, which involves formation and resorption, is an important process for maintaining adequate bone mass in humans. Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammation and bone loss, leading to joint destruction and deformity, and is a representative disease of disrupted bone homeostasis. The bone loss and joint destruction are mediated by immunological insults by proinflammatory cytokines and various immune cells. The connection between bone and immunity has been intensely studied and comprises the emerging field of osteoimmunology. Osteoimmunology is an interdisciplinary science investigating the interplay between the skeletal and the immune systems. The main contributors in osteoimmunology are the bone effector cells, such as osteoclasts or osteoblasts, and the immune cells, particularly lymphocytes and monocytes. Physiologically, osteoclasts originate from immune cells, and immune cells regulate osteoblasts and vice versa. Pathological conditions such as RA might affect these interactions, thereby altering bone homeostasis, resulting in the unfavorable outcome of bone destruction. In this review, we describe the osteoclastogenic roles of the proinflammatory cytokines and immune cells that are important in the pathophysiology of RA.

1. Introduction

Rheumatoid arthritis (RA) is a devastating autoimmune disease characterized by progressive bone destruction. Under physiological conditions, bone remodeling occurs continually, as a coordinated process that results in the formation and degradation of bone. This process is a balance between bone formation, which is mediated by osteoblasts, and bone resorption, which is regulated by osteoclasts, and ensures bone homeostasis. In pathological conditions such as RA, bone homeostasis is disrupted, resulting in uncoordinated osteoclast formation.

Osteoclasts are generated from precursor cells that are usually of the monocyte-macrophage lineage. Interactions between receptor activator of the nuclear factor kappa B (RANK) and its ligand (RANKL) are essential in osteoclastogenesis. RANK on monocyte binds to RANKL, initiating osteoclast differentiation. Under physiological conditions, the main source of RANKL is osteoblasts. However, immune cells and fibroblast-like synoviocytes (FLS) are the main source of RANKL in pathological conditions such as arthritic RA joints (Figure 1). Several systemic and local factors influence the process of osteoclastogenesis. In RA, excessive activation of the immune system could affect the formation and function of osteoclasts. Proinflammatory cytokines tend to be osteoclastogenic; however, the opposite has also been observed [1]. In our literature review, proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-11, IL-17,

and tumor necrosis factor (TNF)-α were frequently reported to be osteoclastogenic, and IL-4, IL-10, IL-13, IL-18, interferon (IFN)-γ, and IFN-β were anti-osteoclastogenic. T cell subpopulations have been studied for their contribution to osteoimmunology. T helper 17 cells (Th17 cells), a specific subtype of T helper cells that produce IL-17 and RANKL, were reported to be osteoclastogenic, whereas the classical Th1 and Th2 cells were generally reported to be anti-osteoclastogenic through their production of IFN-γ (Th1) and IL-4 (Th2) [2, 3].

We could not draw uniform conclusions about the various factors involved in osteoclastogenesis. Some proinflammatory cytokines, such as IL-7, IL-12, IL-23, and TGF-β, possess dual osteoclastogenic and anti-osteoclastogenic properties. Their net effect depends on the specific pathophysiological conditions in *in vivo* models, whereas it depends on the developmental stage of the osteoclasts [4–6] in *in vitro* experiments. The determination of their exact role in the bone microenvironment is even more difficult because these cytokines can have synergistic or antagonistic effects on osteoclasts [7–11].

The joint structure is invaded and the bone is destroyed by the pannus, which contains a massive infiltration of immune cells, proliferative vessels, and increased numbers of osteoclasts (Figures 2(a) and 2(b)). These complicated structures are frequently observed in RA at the synovium-bone interface (Figure 2(c)). This review will address immune-mediated bone destruction in two sections. First, the osteoclastogenic role of proinflammatory cytokines will be discussed. In the following section, the osteoclastogenic role of inflammatory cells that play important roles in the pathogenesis of RA will be described.

2. Cytokines and Bone: The Osteoclastogenic Effect of Proinflammatory Cytokines

Proinflammatory cytokines promote osteoclastogenesis via RANKL expression. Some researchers have shown that proinflammatory cytokines such as TNF-α, IL-1, and IL-6 are capable of inducing osteoclast differentiation independently of RANKL [12–14]. Others showed that a minimal level of RANKL is essential for TNF-α-induced osteoclastogenesis, revealing that TNF-α alone does not induce osteoclast formation [15]. To clarify this controversy, we adopted a simplified monocellular culture system instead of a co-culture system, which consists of osteoblasts and bone marrow cells [16]. In our experience, permissive levels of RANKL were required for cytokine-associated osteoclastogenesis. IL-1 increased and IL-6 decreased the number of mature osteoclasts in a dose-dependent manner. Treatment with IL-23, IL-17, or TNF-α resulted in various responses according to the exposure time and the cytokine concentration.

The effects of important cytokines on osteoclastogenesis *in vitro* and *in vivo* are summarized in Table 1. Based on laboratory observations, cytokine-targeting therapies were tested in bone resorptive conditions. The results of experimental and clinical trials are presented in Table 2.

2.1. TNF-α. TNF-α has received attention from immunologists and rheumatologists because several TNF-α inhibitors show enormous pharmaceutical success in treating RA. TNF-α is produced by activated T cells and is involved in inflammation- and cancer-induced bone loss [17]. Treatment with TNF-α inhibitors results in decreased inflammation and bone protection in RA patients [18]. *In vivo* blockade of TNF-α reduces bone resorption in postmenopausal osteoporosis [19]. Thus, TNF-α is regarded as a major contributor to bone destruction and osteoclast formation.

TNF-α promotes bone destruction by upregulating the production of RANKL and macrophage colony-stimulating factor (M-CSF) from osteoblasts and stromal cells, and by augmenting differentiation into osteoclasts independently of RANK-RANKL signaling [20]. In addition, TNF-α and RANKL synergistically upregulate the expression of RANK [21]. This osteoclastogenic effect of TNF-α is closely associated with other inflammatory cytokines, including IL-1 and M-CSF [22–24]. Although osteoclastogenesis is a more dominant mechanism in the bone erosion of inflammatory disease, osteoblast formation is also affected by TNF-α. TNF-α inhibits osteoblast differentiation primarily through TNF-receptor 1 signaling [25, 26].

2.2. IL-1. IL-1, a proinflammatory cytokine, is highly expressed in patients with RA [27]. An earlier study showed a prominent protective effect of IL-1 blockade against structural damage in an arthritis animal model, suggesting a crucial effect of IL-1 on bone metabolism [28]. Animal models with a deficiency of IL-1 signaling present with reduced osteoclastogenesis, leading to significantly increased levels of bone density, trabecular bone mass, and cortical thickness [29, 30]. IL-1 also plays an important role in the bone loss induced by estrogen deficiency; the level of IL-1 increases after menopause and decreases with estrogen replacement [31, 32]. Bone resorption is suppressed by blockade of IL-1 in postmenopausal women [19].

IL-1 induces RANKL to promote osteoclastogenesis through the production of prostaglandin E in periodontal tissue [33, 34]. Furthermore, IL-1 might exert a bone resorptive effect via an alternative pathway independent of the RANK/RANKL signal [35, 36]. IL-1 is essential for TNF-α-induced osteoclastogenesis. Human TNF-α transgenic mice lacking IL-1β were protected from systemic bone loss regardless of sustained inflammation [37]. The activation of p38 mitogen-activated proteinase kinase is involved in TNF-α- and IL-1-mediated osteoclastogenesis by upregulating RANKL expression in stromal cells and stimulating osteoclast precursor differentiation [23].

2.3. IL-6. Dysregulation of IL-6 is frequently observed in RA patients [38–40]. IL-6 is responsible for synovial inflammation as well as the structural damage of RA. An IL-6 receptor antagonist, a new immunotherapeutic, reduced bone turnover, favoring bone protection in RA patients [41, 42]. IL-6 is also involved in other diseases associated with accelerated bone turnover, such as multiple myeloma and Paget's disease of bone [43].

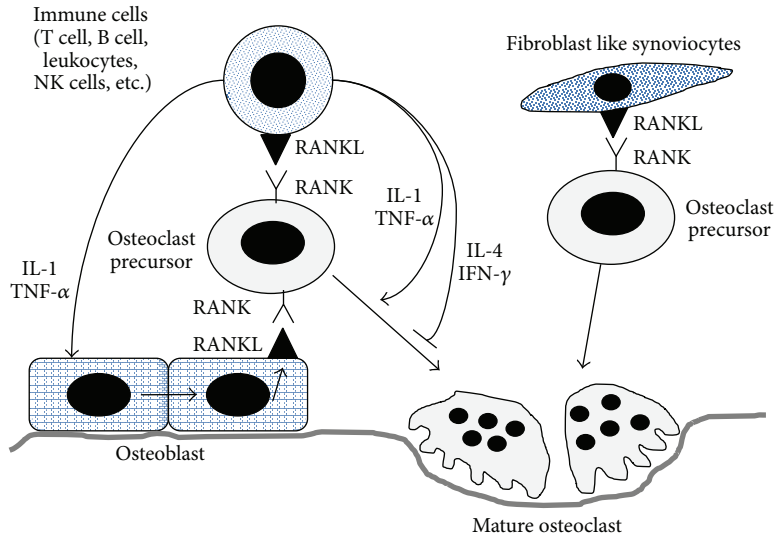

Figure 1: Osteoblast-derived RANKL binds to RANK on monocytes to differentiate them into mature osteoclasts. Osteoblast-derived RANKL plays important role in generating osteoclast in physiological condition. However, immune cell and FLS-derived RANKL play pathogenic role in RA. Proinflammatory cytokines such as IL-1 and TNFα effectively stimulate osteoblast to express RANKL. FLS-derived RANKL enhances osteoclastogenesis in RA joints. RANK: receptor activator of the nuclear factor kappa B; RANKL: receptor activator of the nuclear factor kappa B ligand; FLS: fibroblast like synoviocyte; RA: rheumatoid arthritis; IL-1: Interleukin-1; TNFα: tumor necrosis factor-alpha.

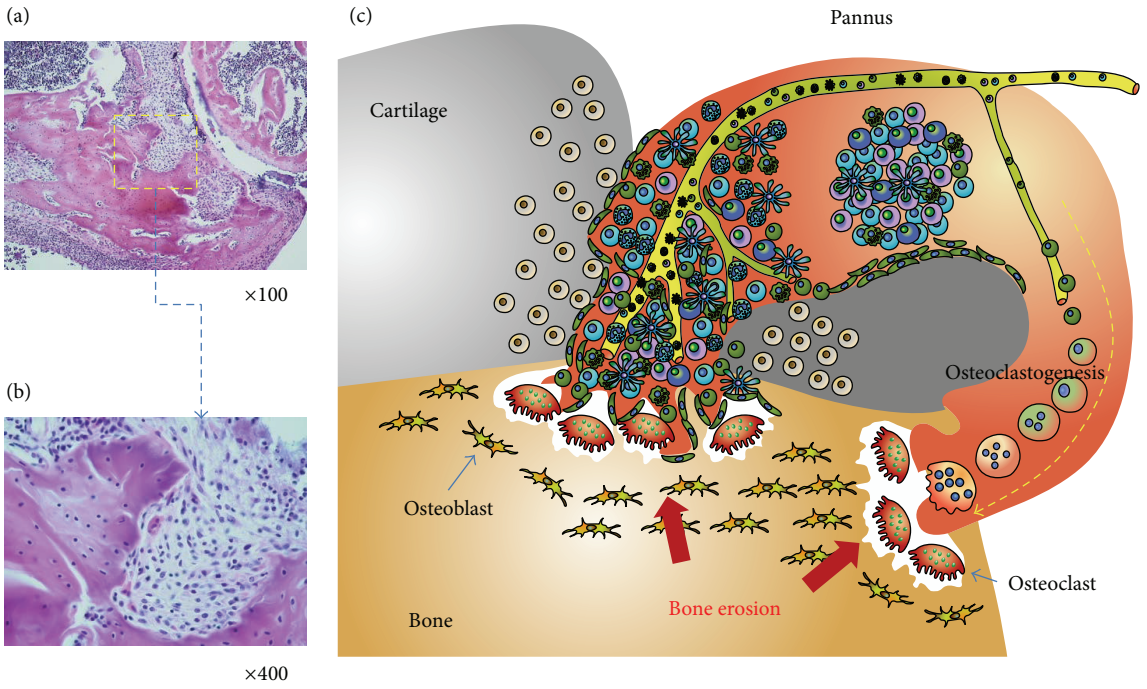

Figure 2: (a) Bone is destroyed by a proliferative and invasive synovium, which is called pannus. It originates from adjacent synovial tissue and invades the cartilages and bones. (b) Magnified view of the pannus-bone interface. The pannus-bone interface is lined with mature osteoclasts (arrows). Various inflammatory cells and stromal cells comprise the invading pannus. (c) Schematic depiction of the pannus-cartilage-bone structure. Inflammatory cells such as B cells, T cells, macrophages, monocytes, and fibroblast-like synoviocytes accumulate in the pannus. For metabolic support, intensive angiogenesis is usually followed. Excessive provision of RANKL from the accumulated cells in the pannus enhances osteoclastogenesis, resulting in the erosion of bone at the pannus-bone interface.

Table 1: Roles of cytokines on osteoclastogenesis.

	In Vitro	*In Vivo*
TNF-α	Osteoclastogenic (i) Upregulates the expression of RANKL and osteoclast activators (ii) Enhances osteoclast differentiation synergistically with RANKL or independently of RANKL (iii) Inhibits osteoclast apoptosis References: [12, 21, 110–116]	Osteoclastogenic (i) Upregulates the expression of RANKL and osteoclast activators (ii) Induces osteoclastogenesis in the presence or absence of RANKL (iii) Plays a critical role in inflammatory arthritis (iv) Associated with estrogen-deficient osteoporosis and joint destruction in RA References: [15, 24, 36, 117–119]
IL-1	Osteoclastogenic (i) Upregulates the expression of RANKL and osteoclast activators (ii) Enhances osteoclast differentiation synergistically with RANKL or independently of RANKL References: [33, 35, 116, 120–122]	Osteoclastogenic (i) Induces osteoclastogenesis in the presence or absence of RANKL (ii) Mediates TNF-α-induced osteoclastogenesis (iii) Participates in physiological bone metabolism (iv) Associated with estrogen-deficient osteoporosis References: [23, 29–32, 36, 37]
IL-6	Osteoclastogenic (i) Upregulates the expression of RANKL and osteoclast activators (ii) Induces RANKL-dependent osteoclastogenesis References: [10, 44, 123–129] Antiosteoclastogenic (i) Suppresses the RANK signaling pathway (ii) Diverts cells into the macrophage lineage References: [6, 133, 134]	Osteoclastogenic (i) Enhances osteoclastogenesis in the prepubertal stage (ii) Supports osteoclastogenesis in callus formation during fracture healing (iii) Associated with bone loss from inflammatory arthritis and estrogen deficiency References: [46, 48, 49, 130–132] Antiosteoclastogenic (i) Suppresses the differentiation of early osteoclast precursor cells (ii) Decreases osteoclast formation, leading to reduced bone turnover References: [46, 47, 135]
IL-17	Osteoclastogenic (i) Induces the expression of RANKL and proinflammatory cytokines (ii) Increases sensitivity to RANKL (iii) Enhances osteoclastogenesis via prostaglandin E2 (PGE2) in osteoblasts References: [1, 53, 136–141] Anti-osteoclastogenic (i) Suppresses osteoclast formation at high concentrations (ii) Inhibits osteoclastogenesis by induction of GM-CSF References: [145, 146]	Osteoclastogenic (i) Induces the expression of RANKL and proinflammatory cytokines (ii) Mediates estrogen-deficient osteoporosis References: [52, 142–144]
IL-23	Osteoclastogenic Induces osteoclastogenesis via IL-17 References: [56] Antiosteoclastogenic Inhibits osteoclast formation via T cells References: [57, 151]	Osteoclastogenic (i) Induces the expression of RANKL (ii) Expands myeloid-lineage osteoclast precursors References: [65, 147–150] Antiosteoclastogenic Limits the resorption of immature bone below the growth plate References: [57]

The previous data indicate the dual functions of IL-6 on bone remodeling. The addition of IL-6 and the soluble IL-6 receptor into bone tissue cultures stimulates bone resorption through increased RANKL expression on osteoblasts [44] via activation of the STAT3 pathway [45]. However, IL-6 exhibits a direct inhibitory effect on RANK signaling in osteoclast progenitor cells in the absence of other supporting cells [6].

In vivo studies also suggest that the role of IL-6 varies in a context-dependent manner. IL-6 transgenic mice with a high level of circulating IL-6 exhibited enhanced osteoclastogenesis, leading to impaired skeletal growth at the prepubertal stage [46] but decreased osteoclast formation at the adult stage [46, 47]. Under physiological conditions, IL-6 deficiency resulted in no detectable change in osteoclast

Table 2: Effects of biologic therapies on bone.

	Mice	Human
TNF-α blockers	Bone-protective in inflammatory arthritis and estrogen deficiency	Bone-protective in inflammatory disease Changes in bone turnover markers in postmenopause (small observational study)
	References: [152–158]	References: [17, 19, 20, 159]
IL-1 blockers	Bone-protective in inflammatory arthritis and estrogen deficiency	Bone-protective in RA (not usually recommended; less effective than other biologic agents) Changes in bone turnover markers in postmenopause (small observational study)
	References: [28, 155, 157]	References: [19, 160, 161]
IL-6 blockers	Bone-protective in inflammatory arthritis No effects in estrogen deficiency	Bone-protective in RA
	References: [155, 156, 162, 163]	References: [41, 42, 164, 165]
IL-17 blockers	Bone-protective in inflammatory arthritis and estrogen deficiency	No data in bone metabolism
	References: [55, 138, 144, 166, 167]	
IL-23 blockers	Bone-protective in inflammatory arthritis	No data
	References: [56]	

number [48]. However, IL-6 knockout mice were protected against ovariectomy-induced bone loss [48]. IL-6 knockout mice with experimental arthritis showed significantly decreased osteoclastogenic activity and impaired osteoclast recruitment to inflammatory sites [49]. These results indicate that IL-6 is associated with bone loss from inflammation and estrogen deprivation. IL-6, along with TGF-β, induces the differentiation of naïve T cells into Th17 cells, which are typically osteoclastogenic [50].

2.4. IL-17. IL-17 is predominantly expressed by Th17 cells, a specific type of human T helper cells [51]. It is hypothesized that this cytokine plays a crucial role in inflammation and the development of autoimmune diseases, including RA. There is evidence that IL-17 enhances osteoclastogenesis by a RANKL-RANK dependent mechanism. Studies of an arthritis animal model indicate that IL-17 induces the expression of RANKL and proinflammatory cytokines such as IL-1 and TNF-α [52]. These inflammatory mediators (IL-17, IL-1, TNF-α, and RANKL) interact with each other in the progression of RA. IL-17A also upregulates the expression of RANK on osteoclast precursors and increases their sensitivity to RANKL [53]. Similarly, treatment with an IL-17 neutralizing antibody inhibited bone destruction in collagen-induced arthritis [54, 55]. However, the mechanisms of action of IL-17 in bone erosion remain to be determined, particularly in association with other osteoclastogenic cytokines such as IL-1, TNF-α, and RANKL.

2.5. IL-23. One of the most important stimuli for IL-17 synthesis is IL-23 produced by activated dendritic cells and macrophages [50]. IL-23 is implicated in inflammatory diseases, in association with IL-17. Accordingly, the IL-23/IL-17 axis plays a critical role in controlling inflammatory bone loss. Recent work suggests that osteoclastogenesis is promoted by IL-23 and inhibited by an anti-IL-23 antibody [56]. By contrast, another study shows the indirect inhibition of osteoclast differentiation by IL-23 *in vitro*. Under physiological conditions, IL-23 promotes higher bone mass in long bones by limiting bone resorption near the growth plate *in vivo* [57]. These conflicting data suggest different roles for this cytokine in physiological or inflammatory bone turnover.

3. Immune Cells and Bone: The Osteoclastogenic Effect of Inflammatory Cells

Various immune cells play important roles in the pathogenesis of RA. These cells comprise the rheumatoid synovium that is continuously inflamed and invades adjacent tissue, resulting in joint destruction (Figure 2). Although osteoclasts are the final effectors of bone erosion, osteoclastogenesis is regulated by various cells in the RA synovium. FLS are the main cellular component of the matrix that is involved in bone turnover. Monocytes, T cells, B cells, and neutrophils also infiltrate the RA synovium and interact with each other. These cells vigorously contribute to osteoclast formation under inflammatory conditions by producing osteoclastogenic cytokines or RANKL (Figure 3).

3.1. Fibroblast-Like Synoviocytes (FLS). Under physiological conditions, the synovium secretes synovial fluid and provides mechanical stability to the joint. However, pathological conditions such as RA render the synovium more aggressive. The synovium forms a pannus with inflammatory cells, enabling invasion into the bone [58, 59]. Histopathology demonstrates increased bone resorption at the bone-pannus interface in the joints of patients with RA. Thus, FLS play an active role in the pathogenesis of RA [59].

The bone and cartilage destruction in RA patients is partly mediated by metalloproteinases secreted by activated synoviocytes and chondrocytes [60, 61]. More importantly, bone destruction is further exacerbated by osteoclasts induced by the RA synovium [62, 63]. We reported that RANKL is produced by FLS from RA patients (RA-FLS) and that osteoclasts are formed in cocultures of RA-FLS and human monocytes [64]. Consistent with a previous report [62], this result indicates that RA-FLS have the capability to support osteoclast differentiation. In RA, FLS upregulate the expression of RANKL and osteoclastogenic cytokines. Earlier studies show that RANKL in RA-FLS can be increased by IL-23 [65], IL-22 [66], and SDF-1 [67]. Furthermore, FLS produce osteoclastogenic cytokines such as IL-6 in response to IL-17 and IL-23 [68, 69]. These inflammatory mediators from stimulated RA-FLS act on stromal cells to upregulate RANKL expression and on osteoclast precursor cells to promote differentiation into osteoclasts (Figure 4).

3.2. Monocyte and Dendritic Cells. Bloodstream monocytes migrate into inflammatory tissue where they differentiate into resident macrophages and dendritic cells (DCs) [70]. Macrophages and DCs express a variety of inflammatory cytokines involved in the pathogenesis of RA [71].

Synovial macrophages play a central role in rheumatoid inflammation. TNF-α, IL-1, and IL-6 are largely produced by activated macrophages and synovial fibroblasts in the RA synovium [71, 72]. As discussed above, these cytokines directly exert osteoclastogenic effects, either synergistically with RANKL or independently of the RANKL signaling pathway. Moreover, macrophages in the RA synovium also secrete TGF-β, IL-21, and IL-23 to differentiate CD4+ T cells into Th17 cells, which are typically referred to as osteoclastogenic T cells.

DC, highly differentiated antigen-presenting cells, interact with T cells and B cells in RA. The physiological function of DC in bone remodeling appears to be modest, as DCs are not frequently observed in bone or the adjacent stroma under normal conditions. By contrast, active lesions of RA and periodontitis retain mature and immature DCs [73–78]. At these sites, DCs contact and interact with T cells to elicit inflammatory processes that involve RANK-RANKL signaling [77]. In multiple myeloma, DCs promote osteoclastogenesis, leading to bone destruction, possibly by activation of RANK-RANKL signaling [79] and the overproduction of IL-17 [80].

DCs can also affect bone metabolism in a more direct manner. Rivollier and colleagues showed that human monocyte-derived DCs transdifferentiate into osteoclasts in the presence of M-CSF and RANKL *in vitro*, suggesting that DCs might directly contribute to osteoclastogenesis [81]. Alnaeeli et al. tested whether the interaction between DCs and T cells supports osteoclast development using an *in vitro* co-culture system of bone marrow-derived CD11c+ DC and CD4+ T cells. Murine CD11c+ DC developed into functional osteoclasts after interactions with CD4+ T cells and stimulation with microbial or protein antigens. Adoptive transfer of DC-derived osteoclasts could induce bone resorption in NOD/SCID mice calvarias *in vivo* [82]. The differentiation of DCs into osteoclasts is frequently reported in the pathogenesis of multiple myeloma [79, 83].

3.3. T Cells. T cells are one of the key regulators of synovial inflammation in RA, having both stimulatory and inhibitory roles [71]. T cells can also play a destructive or a protective role in bone metabolism in a context- and subtype-dependent manner.

In the resting state, T cells seem to have a positive effect on bone mineral density, as T cell depletion increased osteoclastogenesis *in vitro* [84] and accelerated bone resorption *in vivo* [85]. T cell-deficient nude mice have significantly higher numbers of osteoclasts and reduced bone density compared to controls [85].

In response to antigenic stimuli, CD4+ T cells differentiate into distinct effector subsets, Th1 and Th2 cells, which are classically defined on the basis of cytokine production profiles [86]. Th1 cells are characterized by the secretion of IFN-γ, IL-2, IL-12, TNF-α, and TNF-β, and are involved in the elimination of intracellular pathogens [87]. Th2 cells produce IL-4, IL-5, IL-6, IL-9, and IL-13, and are responsible for parasite eradication and allergic disorders [87, 88]. In one comprehensive study, Th1 and Th2 cells were shown to inhibit osteoclastogenesis through IFN-γ and IL-4, respectively [89]. However, the bone-preserving effects of Th1 and Th2 cells are not certain, because contradictory responses have been observed in inflammatory conditions. Infection and inflammation could activate T cells to produce osteoclastogenic cytokines such as TNF-α and RANKL. In the pathogenic state, lymphocytes express significantly higher levels of RANKL and have the capacity to induce RANKL-dependent osteoclast differentiation, unlike in healthy conditions [90]. In addition, IFN-γ exerts a bone resorptive effect instead of a bone-protective effect in an animal model with ovariectomy, infection, and inflammation [4, 91]. Thus, further research is required to understand the net effect of Th1/Th2 cells in disease states such as RA.

Th17 cells, a more recently characterized subset of CD4+ T cells, have been shown to be more osteoclastogenic. Th17 cells are produced when naïve T cells are activated by TGF-β and IL-6 in mice or TGF-β and inflammatory cytokines in humans [50, 92]. Th17 cell play a pivotal role in the pathogenesis of RA through the production of Th17 signature cytokines [50]. Since IL-17 is predominantly produced by Th17 cells and is closely associated with osteoclastogenesis, Th17 cells are likely to affect bone metabolism primarily through IL-17. IL-17 directly induces the expression of RANKL from surrounding cells and facilitates the recruitment of inflammatory cells, leading to an abundance of inflammatory cytokines such as TNF-α and IL-1. Moreover, Th17 cells drive RA-FLS to produce IL-6, IL-8, and matrix metalloproteinases, which potentiate structural damage [93]. A prominent role for Th17 cells has been demonstrated in bone destructive diseases such as RA and multiple myeloma [94, 95] (Figure 5).

3.4. B Cells. Multiple myeloma is a malignant B cell disease characterized by multiple bone lesions. These are caused by

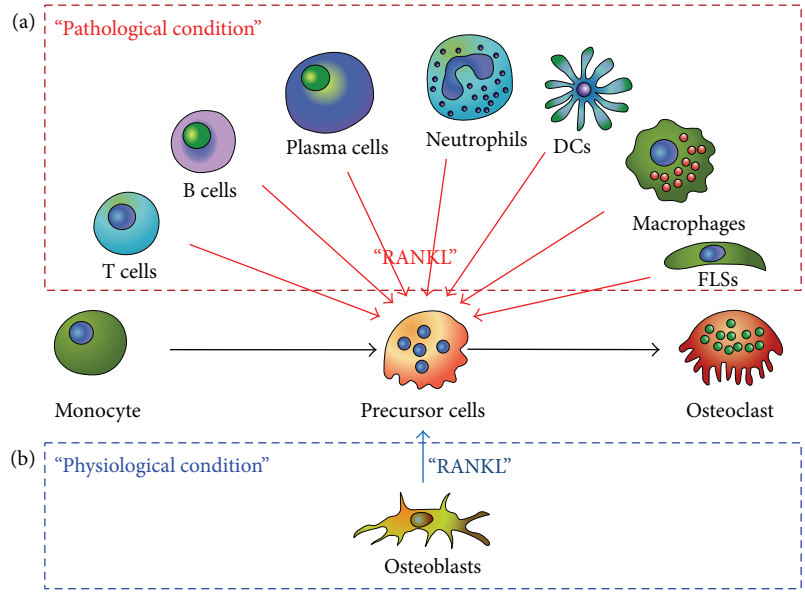

Figure 3: (a) Monocytes are differentiated into mature osteoclasts by the aid of RANKL. In pathological conditions such as inflammation, cancer, and hypermetabolism, various cells extraordinarily provide RANKL to the monocytes, resulting in overweighed osteoclastogenesis. In this condition, the osteoclasts outnumber the osteoblasts, disrupting the bone homeostasis. Bone erosion or osteoporosis is the major outcome of disrupted homeostasis. (b) In normal physiological conditions, a few cells, predominantly osteoblasts, express RANKL. A similar number of osteoclasts and osteoblasts maintain the bone mass by homeostatic equilibrium.

plasma cells expressing RANKL, which stimulate osteoclast formation, leading to osteolysis [96]. This phenomenon indicates that B cells could affect bone metabolism via RANKL expression. In RA, the pathophysiologic role of B cells is highlighted by the therapeutic success of B cell-depleting therapy with an anti-CD20 monoclonal antibody (rituximab) [97, 98]. B cells play an important role in producing autoantibodies. Although the role of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibody is not fully understood, these autoantibodies are associated with more severe bone destruction [99]. Treatment with rituximab reduced bone destruction as well as joint inflammation. Taken together, these findings indicate that B cells contribute to bone destruction through RANKL expression and the production of autoantibodies in cooperation with other immune cells.

3.5. Neutrophils. The neutrophil is the most abundant type of white blood cell in mammals, and comprises an essential part of the innate immune system. Neutrophils normally circulate in the bloodstream and migrate to the site of inflammation in response to inflammatory stimuli. In the RA synovium, neutrophils regulate inflammation through the secretion of inflammatory mediators [100]. Histological analysis of bony lesions in humans and animal models indicates the involvement of neutrophils in pathogenic bone remodeling. Infiltration of neutrophils is observed in human periodontitis and experimental arthritis [101–103]. The RANKL-RANK-osteoprotegerin pathway is upregulated in activated neutrophils from inflammatory sites [104]. Membrane RANKL on neutrophils is strongly overexpressed after stimulation with lipopolysaccharide and thus mediates osteoclastic bone resorption through the interactions between neutrophils and osteoclasts [105]. The osteoclastogenic effect of neutrophils could be reproduced with purified neutrophil membranes, but not with culture supernatants from activated neutrophils. Thus, the effect of RANKL in activated neutrophils is predominantly mediated by the membrane-bound form, in contrast to activated T cells, where RANKL signaling is mediated by both cell surface and soluble RANKL [106, 107]. In addition, neutrophils affect the function of osteoblasts in children on chronic glucocorticoid therapy and in patients with tophaceous gout, resulting in altered bone remodeling [108, 109].

4. Conclusions

The human body attempts to maintain bone mass in order to maintain skeletal strength. Bone mass is not static but dynamic, and results from the formation or resorbtion of the bony matrix by osteoblasts or osteoclasts. In pathological states such as RA, in which bone resorption is favored over bone formation, osteoblasts are outnumbered by osteoclasts. Osteoclastogenesis is also favored over osteoblastogenesis by the inflammatory milieu. Recent studies have shown that numerous cytokines and immune cells have osteoclastogenic effects, although their exact roles in pathological states are difficult to determine because of the complexity of immune networks in the human body. Proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-17 tend to be osteoclastogenic. The immune cells that participate in the pathogenesis of RA often enhance osteoclastogenesis by

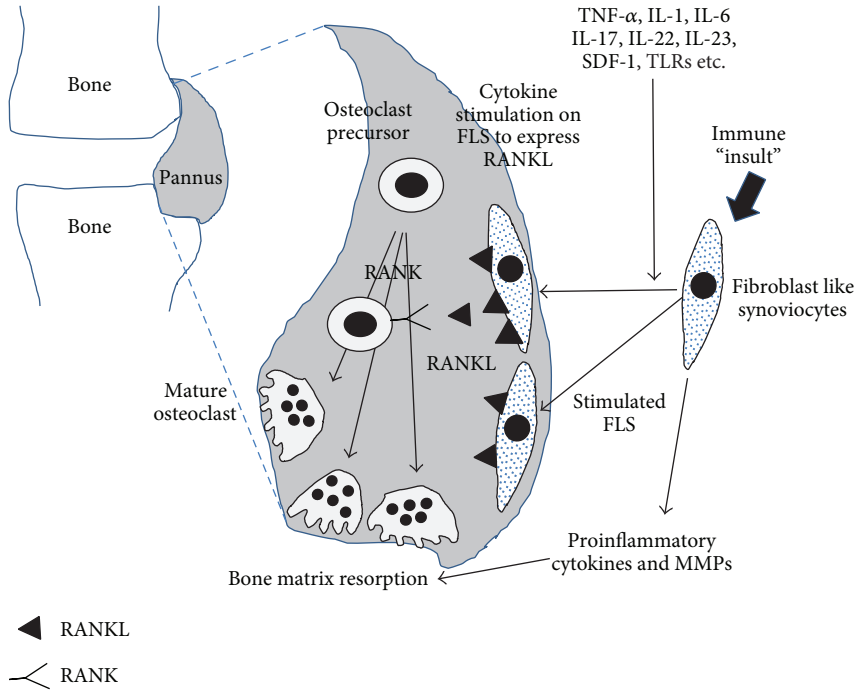

Figure 4: FLS could express RANKL in response to various stimuli. FLS-expressed RANKL enhances osteoclastogenesis and results in bone erosion in RA. Inflammatory and immune stimulation induce the FLS to produce proinflammatory cytokines and matrix metalloproteinase. These cytokines and enzymes aid osteoclasts to destroy the bone matrix.

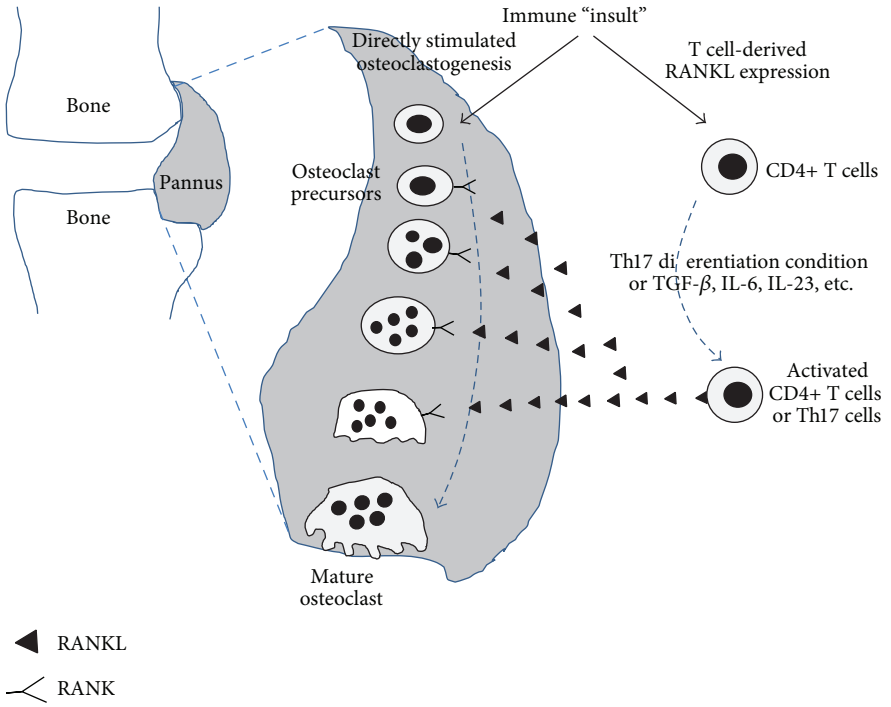

Figure 5: T cells are activated to produce RANKL or osteoclastogenic cytokines by various stimuli. RANKL and activated T cell-cytokines have the potential to induce osteoclastogenesis. With T cells, the outnumbered osteoclasts destroy the bone in RA.

upregulating RANKL directly or by secreting proinflammatory cytokines that influence RANKL expression indirectly. Understanding the precise mechanisms of immune-mediated bone destruction would increase opportunities for target-specific inhibition of bone erosion or osteoporosis. Therapeutic interventions specifically targeting osteoclastogenesis might enable clinicians to spare bone mass in RA patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publishing of this paper.

Authors' Contribution

Seung Min Jung and Kyoung Woon Kim equally contributed.

Acknowledgments

This work was supported by Grants from the Korea Healthcare Technology R&D Project of the Ministry for Health, Welfare, and Family Affairs, Republic of Korea (A092258); from the Basic Science Research Program of the Ministry of Science, ICT, and Future Planning of the National Research Foundation of Korea (2013R1A1A1076125); and from the Advanced Production Technology Development Program of the Ministry for Food, Agriculture, Forestry, and Fisheries (312037-05).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 943149, 8 pages
http://dx.doi.org/10.1155/2014/943149

Review Article

SOCS1 and Regulation of Regulatory T Cells Plasticity

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Received 4 February 2014; Revised 10 June 2014; Accepted 30 June 2014; Published 15 July 2014

Academic Editor: Mizuko Mamura

Several reports have suggested that natural regulatory T cells (Tregs) lose Forkhead box P3 (Foxp3) expression and suppression activity under certain inflammatory conditions. Treg plasticity has been studied because it may be associated with the pathogenesis of autoimmunity. Some studies showed that a minor uncommitted Foxp3$^+$ T cell population, which lacks hypomethylation at Treg-specific demethylation regions (TSDRs), may convert to effector/helper T cells. Suppressor of cytokine signaling 1 (SOCS1), a negative regulator of cytokine signaling, has been reported to play an important role in Treg cell integrity and function by protecting the cells from excessive inflammatory cytokines. In this review, we discuss Treg plasticity and maintenance of suppression functions in both physiological and pathological settings. In addition, we discuss molecular mechanisms of maintaining Treg plasticity by SOCS1 and other molecules. Such information will be useful for therapy of autoimmune diseases and reinforcement of antitumor immunity.

1. Introduction

Dysregulation of immune tolerance to self causes a variety of autoimmune diseases. In the thymus, tolerance is maintained by the so called "negative selection," deletion of self-reactive T cells. Peripheral tolerance is maintained by the regulatory cells including regulatory T cells (Tregs) [1–4]. Most Tregs mature in the thymus under the influence of relatively high avidity interactions between T cell receptor (TCR) and autoantigens, which are called thymus-derived naturally occurring Tregs (nTregs or tTreg), while some are induced from naïve T cells in the periphery. Tregs consist of 5–10% of CD4$^+$ T cells, which express the transcription factor Forkhead transcription factor (Foxp3) in both humans and mice [1]. Foxp3 plays an essential role in the suppressive functions of Tregs [5], and Foxp3 deficiency causes multi-organ autoimmune diseases such as those observed in the scurfy mouse and in patients with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) [6, 7]. Foxp3$^+$ Tregs can also be generated from naïve T cells by TCR stimulation in the presence of TGFβ and IL-2, which are known as induced Tregs or peripheral Tregs (iTregs or pTregs) [8, 9]. Although iTregs and nTregs have similar suppression activity *in vitro*, Foxp3 expression of iTregs has been shown to be unstable *in vivo* [10]. Recently, it has been shown that the terminally differentiated Tregs are not defined entirely by Foxp3 expression, and the natural Foxp3$^+$ T cell population is heterogeneous, consisting of a committed Treg lineage and an uncommitted subpopulation with developmental plasticity [11]. This uncommitted subset of Tregs has been shown to lose Foxp3 expression rapidly upon transfer into a lymphopenic host [11] or under inflammatory conditions [12]. This phenomenon, called "Treg plasticity," has received much attention, because it may play an important role in the pathogenesis of autoimmunity. For example, Komatsu et al. reported that Th17 cells originating

from Foxp3$^+$ T cells have a key role in the pathogenesis of autoimmune arthritis [13]. Thus, a better understanding of this mechanism is required in order to develop an efficient Treg transfusion therapy for patients with autoimmunity.

In this paper, we review the following: (1) Foxp3$^+$ T cell plasticity, particularly under inflammatory conditions, (2) the effect of suppressors of cytokine signaling 1 (SOCS1) deficiency on Foxp3$^+$ T cell plasticity, and (3) the effect of Foxp3$^+$ T cell plasticity on the possible pathogenesis of autoimmunity, such as systemic lupus erythematosus (SLE).

2. Factors Required for Foxp3 Expression

nTregs develop from progenitor CD4$^+$CD8$^+$ double-positive (DP) T cells as do other single-positive (SP) T cells. TCRs of nTregs are hypothesized to be autoreactive to self-antigens, although Tregs are not deleted [14]. Thus, nTregs are hypothesized to be self-reactive, although no specific self-peptide ligand(s) of an nTreg cell has been identified [15, 16]. In addition to strong TCR signals, the costimulatory receptor CD28 plays an important role in promoting nTreg development. Mice deficient in CD28 or its ligands CD80 and CD86 have significantly reduced nTreg cell populations [17, 18], while deletion of the coinhibitory receptor cytotoxic T lymphocyte antigen (CTLA)-4 results in a higher frequency of nTreg cells [19]. The NF-kB pathway activated by TCR and CD28 plays positive roles in inducing Foxp3, while phosphoinositide 3-kinase (PI3 K) Akt signaling negatively regulates nTreg development [20, 21].

The Foxp3 promoter, which is located 6.5 kb upstream of the first exon, contains six NFAT and AP-1 binding sites as well as a TATA and CAAT box [22]. We recently demonstrated that members of the Nr4a family of nuclear orphan receptors, through their ability to induce Foxp3, are critical in nTreg cell development in the thymus [23, 24]. The promoter is highly conserved between humans, mice, and rats; in addition, three highly conserved noncoding DNA sequences (CNS), CNS1, CNS2, and CNS3, were discovered (Figure 1). CNS1, an intronic enhancer (enhancer 1), contains the TGF-β-responsive elements, that is, the Smad2/3 binding sites, close to the NFAT site. These elements are essential for TGF-β-induced Foxp3 expression in iTreg cells [25, 26]. Genetic deletion of CNS1 in mice revealed that CNS1 is redundant for nTreg cell differentiation, but essential for iTreg cell generation in gut-associated lymphoid tissues [27]. Consistently, naïve T cells lacking both Smad2 and Smad3 could not differentiate into iTregs [28]. CNS2, corresponding to the TCR-responsive enhancer (enhancer 2), contains a CpG island and binding sites for transcription factors, CREB [29] and STAT5 [30]. Zheng et al. demonstrated that CNS2 is required for Foxp3 expression in mature nTreg cells, while CNS3 acts as a pioneer element, playing a prominent role in the generation of nTreg cells in the thymus and the periphery [27]. CNS3 also contains binding sites for transcription factors such as c-Rel [27]. Major transcription factors found to be involved in Foxp3 gene expression are shown in Figure 1.

3. Epigenetic Change in nTregs and Its Role in Treg Stability

Unlike nTregs, TGF-β induced Tregs (iTregs) have been shown to be unstable [31–33]. This unstable phenotype is associated with a strong methylation of the CNS2 region of the Treg-specific demethylated regions (TSDRs) within the Foxp3 locus. This idea is supported by the fact that treatment of iTregs with IL-2/anti-IL-2 complexes in the presence of an antigen stabilized Foxp3 expression while also enhancing demethylation of the TSDR [33].

Foxp3 is essential for the development of regulatory T (Treg) cells, yet its expression is insufficient to establish the Treg cell lineage [34]. A recent study has shown that the coexpression of Foxp3 with at least one of the "quintet factors," namely, the transcription factors GATA-1, IRF4, Lef1, Ikzf4, and Satb1, induces the same pattern of gene expression covering a substantial part of Treg signatures and that this is not achieved by the expression of Foxp3 alone [35]. Ohkura et al. demonstrated that Treg cell development was achieved through a combination of two independent processes, that is, the expression of Foxp3 and the establishment of a Treg cell-specific CpG hypomethylation pattern mostly fond in CNS2 TSDR (nTreg type epigenetics) [36]. This Treg cell-type CpG hypomethylation began in the thymus and spread to the periphery and could be fully established without Foxp3. Hypomethylation of this region was required for Foxp3$^+$ T cells to acquire nTreg cell-type gene expression, lineage stability, and full suppressive activity. Thus, those T cells in which the two events have concurrently occurred are developmentally set into the nTreg cell lineage.

Treg epigenetic components control the Treg-type gene expression patterns, either dependent on, or independently from, Foxp3. A genome-wide comparison of DNA methylation status in conventional CD4$^+$ T cells and Tregs has demonstrated the presence of Treg-specific DNA hypomethylation in the genes that are associated with Treg function [37]; these genes include Foxp3, Foxp3-dependent Treg cell-associated genes (CTLA4 and GITR), and Foxp3-independent Treg cell-associated genes (Helios and Eos) [38].

4. Controversy Surrounding Regulatory T Cell Plasticity

In certain conditions, both murine and human naïve CD4$^+$ T cells transiently express Foxp3, without acquiring a suppressive function [39–41]. Moreover, natural Tregs from the thymus have been shown to convert to effector/helper T cells with a decrease of Foxp3 expression [11]. Such "exFoxp3 cells" [12] or "lapsed Tregs" [42] develop an effector-memory phenotype, produce pathogenic cytokines, and might be a cause of autoimmunity. On the contrary, highly purified Tregs are reported to be stable under both physiologic and inflammatory conditions [43]. Regarding the developmental Treg plasticity, two possible mechanisms have been proposed: (1) committed Foxp3$^+$ cells convert to Foxp3$^-$ cells through lineage reprogramming, and (2) uncommitted Tregs expand and easily lose Foxp3 [44]. A recent study has identified

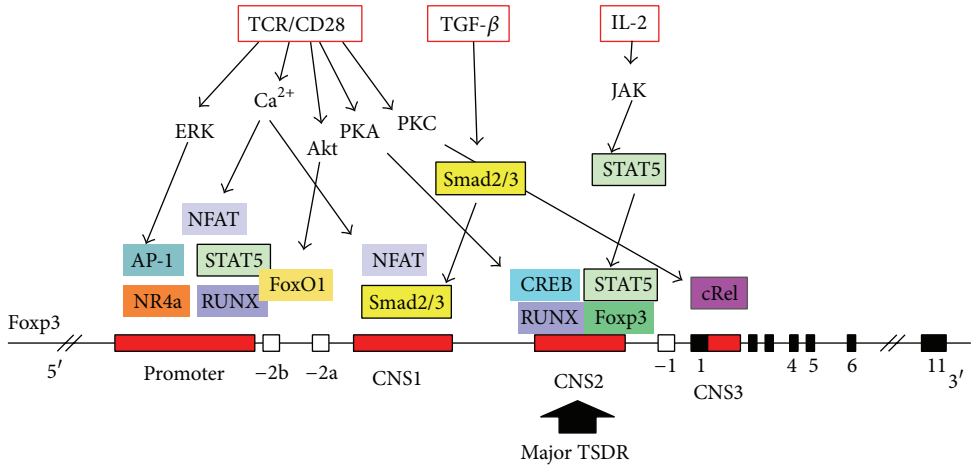

Figure 1: Transcription factors and signals that are involved in Foxp3 induction and stable expression. The promoter and CNSs (conserved noncoding sequences) in the introns are shown. In the course of Treg cell development, epigenetic changes take place and accessibility of CNS2 increases by DNA demethylation, histone modifications, and possibly nucleosome repositioning. The CNS2 region serves as an enhancer for *Foxp3* transcription and is bound by transcription factors such as Foxp3, STAT5, and CREB. These epigenetic alterations are maintained irrespective of environmental changes and thus allow stable *Foxp3* transcription by constitutively expressed transcription factors.

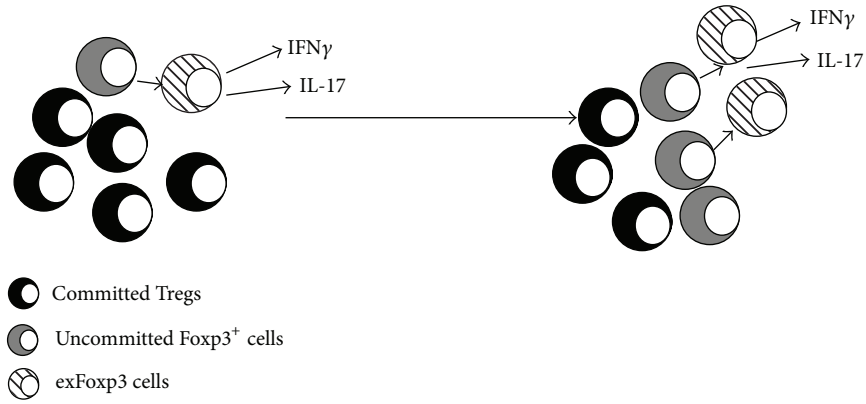

Figure 2: Natural Tregs represent a stable cell lineage; however, there is a minor fraction of Foxp3$^+$ T cells that lack Foxp3 expression, which of conversion to exFoxp3 cells is accelerated under lymphopenic or inflammatory conditions.

a minor uncommitted nonregulatory Foxp3$^+$ T cell population that exhibits transient Foxp3 expression and that lacks TSDR hypomethylation by using fate mapping of mice and methylation analysis [32, 41, 45]. Even committed Tregs are reported to reversibly downregulate Foxp3 expression without losing Treg characteristics [41]. Additionally, CREB and Ets-1 are reported to interact with the CNS2 site of the Foxp3 promoter/enhancer depending on its methylation status and stabilize Foxp3 expression [45–47]. Thus, stable nTregs have a mechanism for stable Foxp3 expression, and a small fraction with unstable Foxp3 expression may contribute to the exFoxp3 population.

In addition to epigenetic modification, protein-protein interactions between Foxp3 and other factors may be involved in Foxp3 stability. Biochemical and mass-spectrometric analyses have revealed that Foxp3 forms complexes with several cofactors [48]. NFAT or Runx1/Cbfβ binds to Foxp3 in Tregs [49–51]. However, it is not very clear how these Foxp3 binding proteins are involved in Treg functional stability [52].

5. Regulatory T Cell Plasticity in Pathological Settings

Conversion of Tregs into pathogenic exFoxp3 cells has been observed under lymphopenic or inflammatory conditions [11, 12, 41, 53–57] (Figure 2). It is important to clarify how exFoxp3 cells that have lost Foxp3 expression can produce proinflammatory cytokines and act as effector cells causing tissue destruction, because there is a high probability that exFoxp3 cells possess autoreactive TCRs [12]. Several reports elucidated that Treg plasticity or IFNγ-producing Foxp3 positive cells might be one of the causes of autoimmune diseases or immunological disorders [12, 58–61].

Recently, lineage reporter and tracer mice and Treg cell transfer have shown the association between Treg plasticity

and autoimmunity, where Tregs possess antigen-specific TCR within the polyclonal repertoire [62]. A substantial fraction of antigen-specific Tregs with features of Foxp3^{high}, CD25^{high}, and demethylation of the TSDR, induces downregulation of Foxp3 transcription, loss of Foxp3 expression, and development of effector and pathogenic T cell characteristics in an experimental autoimmune encephalomyelitis (EAE) model. Additionally, the authors emphasized that exFoxp3 cells were apparent, only when this minor fraction was adoptively transferred into mice with ongoing EAE. Another important study showed that IL-17-expressing exFoxp3 cells were differentiated from CD25^{low} Foxp3^{+}CD4^{+} T cells then accumulated in the inflamed joints in arthritis [13]. Synovial fibroblasts producing IL-6 caused the conversion of Foxp3^{+}CD4^{+} T cells to Th17 cells. These exFoxp3 Th17 cells were elucidated to be more potent osteoclastogenic T cells than conventional Th17 cells derived from naive CD4^{+} T cells, and they expressed Sox4, CCR6, CCL20, IL-23 receptor, and RANKL. Nie et al. also showed that Tregs from RA patients possessed reduced suppression activity due to the dephosphorylation of Foxp3 by TNF-α, which is elevated in human RA patients [63]. TNF-α-induced Treg cell dysfunction is correlated with increased numbers of Th1 and Th17 cells within the inflamed synovium in rheumatoid arthritis. Another study showed the presence of IFN-γ^{+}Foxp3^{+} T cells in MS patients; these double-positive cells acquire a Th1-like phenotype and reduced suppression activity when cultured in the presence of interleukin-12 [58]. These findings establish the pathological importance of a Foxp3^{+} cell subset with unstable Foxp3 in the generation of pathogenic Th cells in human autoimmunity.

6. Factors That Control Treg Stability

A Treg transfusion therapy has been successful in animal models of autoimmunity, and Treg therapies are currently being tried in patients [64]. However, Treg cell instability is a concern for developing a Treg therapy because it could cause unexpected adverse effects in patients, and, thus, the factors leading to Treg stability need to be investigated. There must be a mechanism that prevents the pathogenic conversion of nTregs. As mentioned before, hypomethylation of TSDR is a key factor in the stability of Tregs. In addition, several transcription factors and signaling molecules have been shown to be important for Treg stability. Genetic manipulation of genes specifically in Tregs revealed such genes. Not surprisingly, most of these factors are directly or indirectly involved in Foxp3 transcription.

The deletion of Smad2/3 in nTregs resulted in a rapid loss of Foxp3 expression, suggesting that TGF-β signals may be necessary for maintaining nTregs in inflamed conditions [28]. The IL-2-STAT5 pathway also seems to be very important for the stability of Foxp3, because the loss of Foxp3 and TSDR methylation can be rescued by means of the enhancement of IL-2 receptor signaling with IL-2-anti-IL-2 complex in EAE [65]. The NF-kB pathway is also important for nTreg stability, probably contributing to Foxp3 transcription through c-Rel, since TRAF6 deficiency in nTregs promoted the loss of Foxp3 expression and Th2 type autoimmunity [66]. Inhibition of p300 (Ep300 or KAT3B), a histone/protein acetyltransferase (HAT) in nTreg cells, destabilized Foxp3 expression and impaired nTreg suppressive function [67]. Foxo1 uniquely regulates nTreg stability, not by sustaining Foxp3 expression but by suppressing genes, including the proinflammatory cytokine IFN-γ [68].

Some factors involved in Foxp3 expression have a negative effect. For example, poly (ADP-ribose) polymerase-1 (PARP-1) deficiency in nTregs resulted in stronger suppressive activity and sustained higher expression of Foxp3 and CD25. Thus PARP-1 limits the function of nTregs through modulation of the stable expression of Foxp3 [69]. Deficiencies of C3aR/C5aR signaling augment murine and human iTreg generation, stabilize Foxp3 expression, resist iTreg conversion to IFN-γ/TNF-α-producing effector T cells, and, as a consequence, limit the clinical expression of graft-versus-host disease [70]. Another factor involved in controlling Treg stability is the ubiquitin-dependent degradation of Foxp3. However, the impact of certain protein modifications to Foxp3, such as ubiquitination and acetylation, on nTreg fate and functions remains to be clarified [71].

7. Effects of SOCS1 on Foxp3 Stability and Treg Functions

Inflammatory cytokine signaling including IFN-γ and IL-6 signaling plays important roles in the pathogenic conversion of nTregs. Usually, *bona fide* Tregs are expected to be resistant to the effect of such inflammatory cytokines. SOCS1, an inhibitor of cytokine signaling, plays an essential role in maintaining functional nTregs [72–74]. SOCS proteins are the negative regulators of the cytokine-JAK-STAT pathway [75], and uncontrolled IFNγ signaling results from SOCS1 deficiency. High expression of SOCS1 in Tregs might be linked to a fundamental function of Tregs. SOCS1 deficiency in Tregs did not affect *in vitro* suppression activity, however, impaired suppressive function of Tregs *in vivo* despite the increase in Tregs. SOCS1-deficient Tregs easily lose Foxp3 expression and converted into Th1- or Th17-like cells, probably due to hyperactivation of STAT1 and STAT3. Recently, Ubc13 has been reported to be involved in suppressive activity by controlling effector cytokine signaling molecules of Tregs including SOCS1 [76].

Analysis of T cell-specific-*Socs1*-conditional knockout (*Lck*Cre-*Socs1*$^{f/f}$, *Lck*Cre-cKO) mice has revealed that SOCS1-deficient effector T cells produce high levels of IFNγ and low levels of IL-17 [77]. The defective suppression activity of SOCS1-deficient Tregs from *Lck*Cre-cKO mice was confirmed through the failure to suppress colitis in *Rag2*-deficient mice by the cotransfer of naïve T cells and Tregs. Under lymphopenic conditions, SOCS1-deficient Tregs from *Lck*Cre-cKO mice lost Foxp3 and were converted into Th1 to produce IFNγ with accelerated methylation of DNAs in the CNS2 region of the Foxp3 promoter/enhancer. Foxp3 levels were restored in SOCS1$^{-/-}$IFN$\gamma^{-/-}$Tregs with hypomethylated TSRD.

We propose that STAT1 and STAT3 hyperactivation due to SOCS1-deficiency is the reason for Treg instability and loss

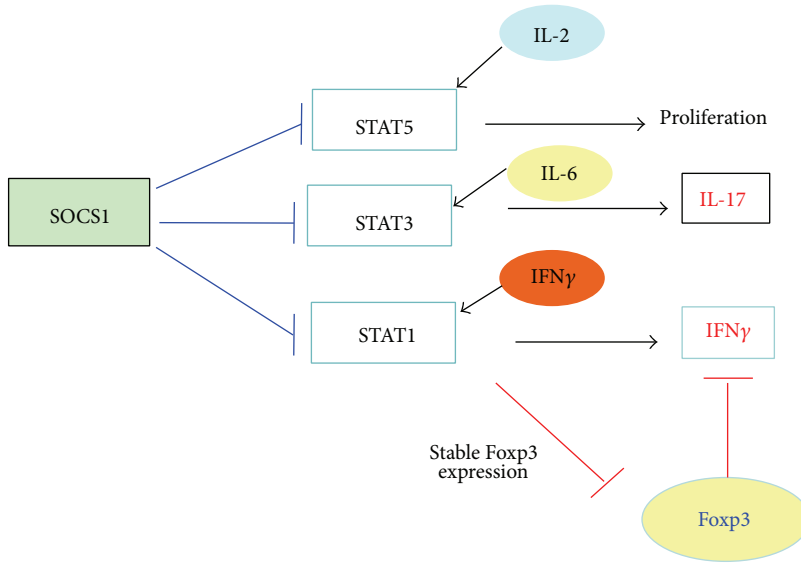

Figure 3: Role of STAT1 and STAT3 in Foxp3 expression and cytokine production in Tregs. SOCS1 protects Tregs from harmful effects of inflammatory cytokines, which promote the loss of Foxp3 expression and the conversion into Th1- and Th17-like effector cells. Hyperactivation of IFN-γ-STAT1 pathway results in the loss of Foxp3 expression and hyperproduction of IFN-γ. Although STAT3 is not directly involved in Foxp3 expression maintenance, it may be crucial for suppression of the production of IL-17. SOCS1-deficient Tregs may expand faster due to hyperactivation of STAT5.

of suppressive functions; however, how activated STAT1 and STAT3 affect Foxp3 expression and Treg functions remained to be elucidated. STAT1 may antagonize STAT5, but this is unlikely because we did not observe a reduction in STAT5 phosphorylation in SOCS1-deficient Tregs, instead SOCS1-deficient Tregs expanded well due to stronger IL-2/STAT5 activity [73]. STAT1 has also been shown to inhibit the TGFβ/Smad pathway [77]. The Smad2/3-deficient Treg phenotypes were similar to those observed in SOCS1-deficient Tregs [28]. Thus, interactive suppression of these molecules by STAT1 may be a mechanism of Foxp3 instability.

Recently, neuropilin-1 (Nrp-1), highly expressed in nTregs but not in iTregs [78, 79], has been implicated in suppressive function of Tregs [28]. Nrp-1 binds semaphorin-4A expressed in mainly plasmacytoid dendritic cells, and this interaction would inhibit Akt-mTOR signaling. High expression of Nrp1 on nTregs might be associated with induction of SOCS1 and Treg plasticity.

We propose the possibility that SOCS1 upregulation in Tregs at appropriate levels maintains Treg functions because SOCS1 may protect Tregs from harmful effects of inflammatory cytokines, which accelerates conversion of Tregs into effector cells (Figure 3).

8. Regulatory T Cell Plasticity in Systemic Lupus Erythematosus (SLE)

SLE is characterized by dysregulated immunity with both hyperactive T cells and B cells, and terminally pathogenic antibodies construct disease conditions. Dysregulation of Treg functions has been implicated in the pathogenesis of SLE. For example, autoreactive T cell expansion and autoantibody production were accelerated in thymectomized or Treg-depleted lupus-prone mice [80]. Transfer of $CD4^+CD25^+$ Tregs from syngeneic normal mice into SLE model mice can effectively suppress the progress of lupus autoimmune phenotypes, such as increased level of ds-DNA antibody and lupus nephritis [81]. We observed that SOCS1-deficient T cells induce lupus-like autoimmunity including spontaneous dermatitis, splenomegaly, and lymphadenopathy with elevated ds-DNA antibodies [57]. Thus, SOCS1 might be important in the pathogenesis of SLE through Treg plasticity.

It is unclear how Treg dysfunction, including Treg plasticity, causes pathogenic autoantibodies with tissue injuries. Studies have reported that adoptive transfer of Tregs into $Cd3\varepsilon^{-/-}$ hosts, which retain B lymphocytes, resulted in the loss of Foxp3 expression and generation of lapsed Tregs that differentiated into follicular helper T cells in Peyer's patches, which promoted IgA class switching [82]. As mentioned, Treg-specific TRAF6-deficient mice possess unstable Tregs and were found to develop SLE-like pathology such as hyperimmunoglobulinemia and anti-dsDNA antibody production [66]. A subset of Foxp3-positive regulatory T cells were recently discovered in the follicular helper T (T_{FH}) cell fraction, so called T_{FR} cells. T_{FR} cells are defined as expressing Foxp3, CXCR5, Bcl-6, and PD-1, localizing in the B cell follicles, and controlling the germinal center reactions to produce IgG [83–85]. TRAF3 was shown to be crucial for antigen-stimulated production of T_{FR} cells to mediate ICOS through NF-kB signaling [86]. However, the origin of T_{FH} cells and whether or not it is associated with Treg plasticity still needs to be clarified.

It has been reported that patients with active SLE have a significantly decreased frequency of activated Tregs, and this decrease is correlated with disease activity [87, 88]. Further research in healthy humans and in patients with autoimmune diseases is required to determine associations between Treg plasticity and SLE.

9. Conclusions

Evidence on associations between Treg plasticity and pathogenesis of autoimmunity including SLE has been reported. We suggest that an important molecule, SOCS1, prevents acceleration of Treg plasticity and development of autoimmunity. However, mechanisms to control Treg plasticity remain to be clarified. There are also few reports on Treg plasticity in humans. A Treg transfusion treatment for autoimmune patients is now being investigated, and it is necessary to determine and control the Treg plasticity mechanisms.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 750374, 6 pages
http://dx.doi.org/10.1155/2014/750374

Review Article

Cellular Factors Targeting APCs to Modulate Adaptive T Cell Immunity

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Received 8 April 2014; Accepted 8 May 2014; Published 14 July 2014

Academic Editor: Mizuko Mamura

The fate of adaptive T cell immunity is determined by multiple cellular and molecular factors, among which the cytokine milieu plays the most important role in this process. Depending on the cytokines present during the initial T cell activation, T cells become effector cells that produce different effector molecules and execute adaptive immune functions. Studies thus far have primarily focused on defining how these factors control T cell differentiation by targeting T cells themselves. However, other non-T cells, particularly APCs, also express receptors for the factors and are capable of responding to them. In this review, we will discuss how APCs, by responding to those cytokines, influence T cell differentiation and adaptive immunity.

1. Introduction

Naïve CD4 T cells stimulated by cognate antigens presented by professional APCs within the lymphoid tissues undergo clonal expansion and differentiate into distinct lineages of effector/regulatory subsets, orchestrate various adaptive immune responses, and then ultimately mature into memory phenotype cells that either continue to circulate the periphery or migrate into nonlymphoid tissues where they play a role in immune surveillance [1, 2]. Extensive efforts have been made to define cellular and molecular mechanisms that initiate the differentiation processes and to identify the features of each T cell subset. While Th1 type cells producing IFNγ are generated in the presence of IL-12, Th2 type cells producing IL-4 and IL-13 are generated in the presence of IL-4. Newly recognized proinflammatory IL-17-producing Th17 type cells are generated in the presence of IL-6 and TGFβ. On the other hand, Foxp3-expressing inducible regulatory T cells can be generated in the presence of TGFβ. Other effector/regulatory T cell subsets include Th9 and Tr1 type cells, which produce IL-9 and IL-10, respectively. Although a great number of cellular and molecular pathways to initiate the differentiation programs have already been uncovered, most of studies have heavily focused on those factors that directly target T cells. In this review, we will focus on the roles of factors that control T cell differentiation processes by acting on non-T-cell targets, especially APCs.

2. APC Stimulation by IL-12 and IFN

Naïve CD4 T cells activated in the presence of IL-12 become Th1 type effector CD4 T cells and play a key role in eliminating intracellular pathogens and viruses. IL-12 is a heterodimeric protein consisting of the IL-12p35 and IL-12p40 subunits [3], mainly produced by antigen presenting cells (APCs), and its production is greatly enhanced by microbial stimuli such as LPS. IL-12 binds the IL-12 receptor (IL-12Rβ1 and IL-12Rβ2) expressed on naive CD4 T cells, signals through STAT4 upon ligation of the receptor [3, 4], and induces transcription of Ets variant gene 5 (ERM) and T-box 21 [5, 6]. T-box transcription factor *Tbx21*, which encodes T-bet, is a Th1 specific transcription factor controlling the expression of

IFNγ [7]. Mice deficient in IL-12 display impaired delayed type hypersensitivity responses and succumb to microbial infections such as *Toxoplasma gondii* or *Cryptococcus neoformans* due to the inability to mount Th1 responses [8, 9]. Likewise, T cells deficient in T-bet are unable to differentiate into Th1 cells and T-bet-deficient mice succumb to pathogen infections that are controlled by Th1 cells [7].

T-bet is also expressed by monocytes and myeloid DCs [10]. Indeed, it was reported that T-bet expression in DCs is essential for optimal induction of Th1 immunity in vivo [11]. T-bet-deficient DCs are unable to produce IFNγ after stimulation and to induce Th1 differentiation [11]. T-bet expression by DCs is also necessary for the Th2 to Th1 repolarization process that occurs during B7-DC cross-linking on DCs [12].

It is evident that IL-12 directly regulates the function of DCs and macrophages. Monocyte-derived DCs express both subunits of IL-12 receptors [13–15]. Stimulation of DCs by IL-12 induces expression of GM-CSF, IL-1β, IL-6, and IFNγ, all of which could have a direct impact on T cell differentiation [16]. IL-12 stimulated DCs or macrophages are also capable of presenting an otherwise poorly immunogenic tumor peptide [14, 17]. An inhibitory mechanism of DC production of IL-12 via DC-specific ICAM-3 grabbing nonintegrin receptor 1 (DC-SIGNR1) was also identified. Stimulating DC-SIGNR1 in DCs increases suppressors of cytokine signaling 1 (SOCS1) and lowers IL-12 production [18].

IFNγ is the signature cytokine produced by differentiated Th1 type effector cells and regulates IL-18R/IL-12R expression on T cells to prime Th1 differentiation [19]. IFNγ also promotes DC maturation and enhances production of cytokines such as IL-1β and IL-12 by directly acting on DCs [20]. IFNγR−/− DCs exhibit impaired function to stimulate alloreactive T cells, to drive Th1 differentiation, and to induce efficient antitumor immunity [21]. Similarly, human monocytes in vitro stimulated with cytokine cocktails consisting of TNFα, IL-1β, IL-6, and prostaglandin E2 produce high levels of IL-10 and low levels of IL-12 [22]. Adding IFNγ into the culture condition dramatically increases IL-12 and decreases IL-10 production, thereby enhancing Th1 polarization [22]. IFNγ induces IL-27 production in DCs and favors induction of IL-10-producing Tr1 cells, limiting Th17-mediated autoimmune neuroinflammation [23]. The protective role of IFNγ is also associated with decreased expression of osteopontin, which is known to have potent proinflammatory function, in DCs [24]. Type I IFN is another potent cytokine similarly influencing adaptive Th17 cell immunity by acting on DCs by regulating expression of IL-27 and osteopontin. IFNα acts on DCs, suppresses intracellular translational isoform of osteopontin, known as Opn-i, enhances IL-27 production, and antagonizes Th17 development [25].

We recently reported that IFNγ signaling in non-T-cell targets plays a critical role in limiting T cell responses and inflammation in the intestine [26]. Using a model of T-cell-induced colitis, a murine model of human inflammatory bowel disease (IBD) induced by naïve CD4 T cells transferred into immunodeficient recipients, we demonstrated that while transfer of naïve wild type CD4 T cells into Rag−/− recipients induces chronic colitis that develops ~4 weeks after transfer, the same CD4 T cells transferred into IFNγR−/− Rag−/− recipients induce acute fulminant colitis within 7 days after transfer [26]. Unlike above-mentioned studies where IFNγ stimulation of DCs alters DC production of cytokines that promote Th1 differentiation, the relative proportion of IFNγ-producing CD4 T cell generation in these recipients is similar in the absence of host expression of IFNγR. Instead, the overall expansion of activated T cells (i.e., IFNγ- and IL-17-producing CD4 T cells) is dramatically enhanced in this condition [26]. It turned out that IFNγ signaling in DCs enhances T cell responses in part by directly controlling survival of DCs. As a result, DCs deficient in IFNγR display enhanced survival in vivo, possibly resulting in prolonged stimulation to T cells [26]. Sercan et al. similarly reported that IFNγ signaling in CD11b+ cells controls memory CD8 T cell differentiation [27]. However, a cellular mechanism by which IFNγ-mediates memory fate decision remains to be examined.

3. APC Stimulation by IL-4

IL-4 is a key cytokine that induces Th2 differentiation by acting on the IL-4 receptors expressed on naïve CD4 T cells [28]. IL-4 signals through STAT6 molecule, activates expression of Th2 associated transcription factors such as GATA3 and c-Maf, and induces expression of Th2 signature cytokines, IL-4, IL-5, and IL-13 [28]. IL-4 exerts its biologic function using two distinct types of IL-4 receptors, type I (IL-4Rα and γc) and type II (IL-4Rα and IL-13Rα1) receptors [29]. The expression of both receptors is different depending on the cell types. While T cells, the major targets of IL-4, express only type I receptors, DCs and macrophages express both receptors [30]. In DCs, differential functions of IL-4 receptors were noted. For example, IL-4 induces DC maturation, upregulating MHCII and costimulatory molecule expression, via type II IL-4R [30]. By contrast, IL-4 also enhances IL-12 production in DCs induced by microbial products, and this function is mediated by the type I IL-4R [30]. IL-4-mediated induction of IL-12 production in DCs is achieved through downregulation of IL-10 [31]. Moreover, IL-4 also induces IL-4 production in DCs [32]. Consistent with these findings, Guenova et al. reported that human DCs differentiated under low-dose IL-4 produce no IL-12 and promote Th2 differentiation, while DCs differentiated under high-dose IL-4 produce large amounts of IL-12 and low IL-10; thereby, Th1 differentiation is preferentially induced [33]. Therefore, IL-4 acting on DCs may influence both Th1 and Th2 differentiation.

Regulatory roles of IL-4 in DC function were recently examined using DC-specific IL-4Rα deficient animals. Hurdayal et al. reported that IL-4 signaling in DCs plays a key role in mounting protective immune responses using a *Leishmania major* infection model [34]. Susceptibility to *L. major* infection is largely determined by the development of Th2 type responses [35]. Paradoxically, IL-4 administration during early infection enhances IL-12 production by DCs and establishes resistance to *L. major* in susceptible BALB/c mice [36]. CD11c-cre IL-4Rα^{flox} mice infected with *L. major* become hypersusceptible to the infection [34].

IL-4-mediated modulation of DC function was also found in other infection models. Infection of IL-4-deficient mice with *Candida albicans* results in impaired development of Th1 immunity [37]. A molecular mechanism underlying the roles of IL-4 in regulating DC production of IL-12 and IL-10 will be a subject of importance especially for vaccine development.

4. APC Stimulation by IL-27

IL-27 is a heterodimeric protein consisting of the IL-27p28 and Ebi3 subunits and binds to the IL-27R complexes made of IL-27Rα/TCCR/WSX-1 and gp130 [38]. IL-27 is primarily produced by APCs activated by IFNγ, TLR ligands, or type I IFNs [38]. Both pro- and anti-inflammatory roles of IL-27 have been well documented. For example, Th1 differentiation is greatly enhanced by IL-27, while IL-27 antagonizes Th1, Th2, and Th17 type effector responses in part by inhibiting IL-2 production [39], [38]. IL-27 induces T-bet expression and activates both STAT1 and STAT4, promoting Th1 differentiation [10, 40]. Accordingly, mice deficient in IL-27Rα −/− infected with intracellular pathogens including *Listeria*, *Leishmania*, and *Mycobacteria* are more susceptible to the infections [41]. Following *Toxoplasma gondii* infection in IL-27Rα −/− mice, a robust IFNγ response is induced and the clearance of the infection is rapidly achieved. However, these mice succumb to uncontrolled immune activation [42], strongly suggesting that IL-27 not only may promote Th1 differentiation, but also may be important to suppress overactive immune responses, possibly by inducing IL-10 by CD4 T cells [43]. IL-27-mediated suppression of Th17 immunity is also supported by the fact that IL-27Rα −/− mice develop more severe experimental autoimmune encephalomyelitis (EAE) [44].

Immunoregulatory roles of IL-27 described above are examples of IL-27 action on T cells. However, IL-27 receptors are expressed on multiple cell types, including DCs and macrophages [38]. IL-27 upregulates MHC and TLR4 expression in human monocytes, resulting in increased production of IL-1β and IL-6 following LPS stimulation [45]. Alternatively, IL-27 downregulates IL-12 production by activated macrophages in *Mycobacterium tuberculosis* infection models [46]. IL-27 is also capable of inducing B7-H1 expression in DCs, which then display reduced function to stimulate allogeneic T cell responses [47]. IL-27 was recently reported to induce CD39 expression in DCs and interferes with EAE development [48]. CD39 induced by IL-27 decreases the extracellular ATP and downregulates ATP-mediated activation of the NLRP3 inflammasome [48].

By contrast, we recently reported that IL-27 signaling in non-T cells especially macrophages and DCs plays a crucial role in generating proinflammatory Th17 responses [49]. This conclusion is made based on the T-cell-induced colitis model using lymphopenic mice deficient in IL-27Rα. While IL-27Rα+ lymphopenic mice that receive naïve CD4 T cells develop chronic colitis associated with colitogenic Th1 and Th17 type effector cell generation, lymphopenic mice deficient in IL-27Rα are completely protected from the colitis after the T cell transfer. Interestingly, generation of IL-17-producing CD4 T cells is completely abrogated in this condition, while IFNγ-producing CD4 T cell generation remains unaltered [49]. To investigate which host cells are responsible for the lack of Th17 differentiation, we next examined DCs and macrophage expression of cytokines promoting Th17 differentiation, namely, IL-1β and IL-6. Indeed, APCs from IL-27Rα −/− mice failed to produce IL-1β and IL-6. Therefore, it seems that IL-27 produced by activated APCs acts on the same or neighboring APCs to increase production of Th17-promoting cytokines. Unlike above-mentioned studies, we were not able to find immunosuppressive roles of IL-27 in DCs. It will be important to identify cellular and molecular pathways of IL-27 leading to immunostimulatory or immunosuppressive function of DCs.

5. APC Stimulation by Other Cytokines

5.1. IL-21. IL-21 is a cytokine produced by activated CD4 T cells and NK cells. Previous studies demonstrated a key role for IL-21 in Th17 differentiation, when acting with TGFβ, as a redundant cytokine to IL-6 [50]. IL-21 also amplifies Th17 differentiation in concert with IL-23 [50–52]. IL-21 is known to have a pleiotropic role by controlling multiple cell functions. IL-21 inhibits DC function to mediate T cell activation [53]. Thus, IL-21-treated DCs are unable to induce CD8 T cell proliferation and contact hypersensitivity responses [53]. It was also reported that IL-21 induces granzyme B expression in human plasmacytoid DCs, which then partially impairs pDC function to stimulate T cell proliferation [54]. Type I diabetes susceptibility locus known as insulin-dependent diabetes susceptibility 3 (*Idd3*) encodes cytokine gene IL-21 and regulates diabetes [55]. It was demonstrated that APCs from diabetes-susceptible NOD and diabetes-resistant NOD. *Idd3* mice differentially support diabetogenic Th17 immunity and that IL-21 signaling in APCs plays a critical role in regulating the Th17-promoting APC functions [55].

5.2. IL-17. IL-17 is the signature cytokine produced by Th17 cells. Many Th17 cells produce IL-17A and IL-17F. IL-17 plays a protective role in host defense against extracellular pathogens and fungus especially at the epithelial and mucosal surface [56]. IL-17 promotes the generation of inflammatory cytokines and chemokines, which attract neutrophils and macrophages to the sites [57, 58]. IL-17-responding targets cells include nonhematopoietic cells including fibroblasts and epithelial cells, as well as macrophages and neutrophils. IL-17 also acts on DCs [59]. IL-17A but not IL-17F stimulates bone marrow derived DCs to secrete more IL-12, IL-6, and IL-1β [59]. IL-17A also upregulates MHCI expression in DCs [59]. IL-17 stimulation in DCs enhances cross-priming function for CD8 T cells during *Listeria* infection [59], although whether it also affects CD4 T cell responses remains to be examined.

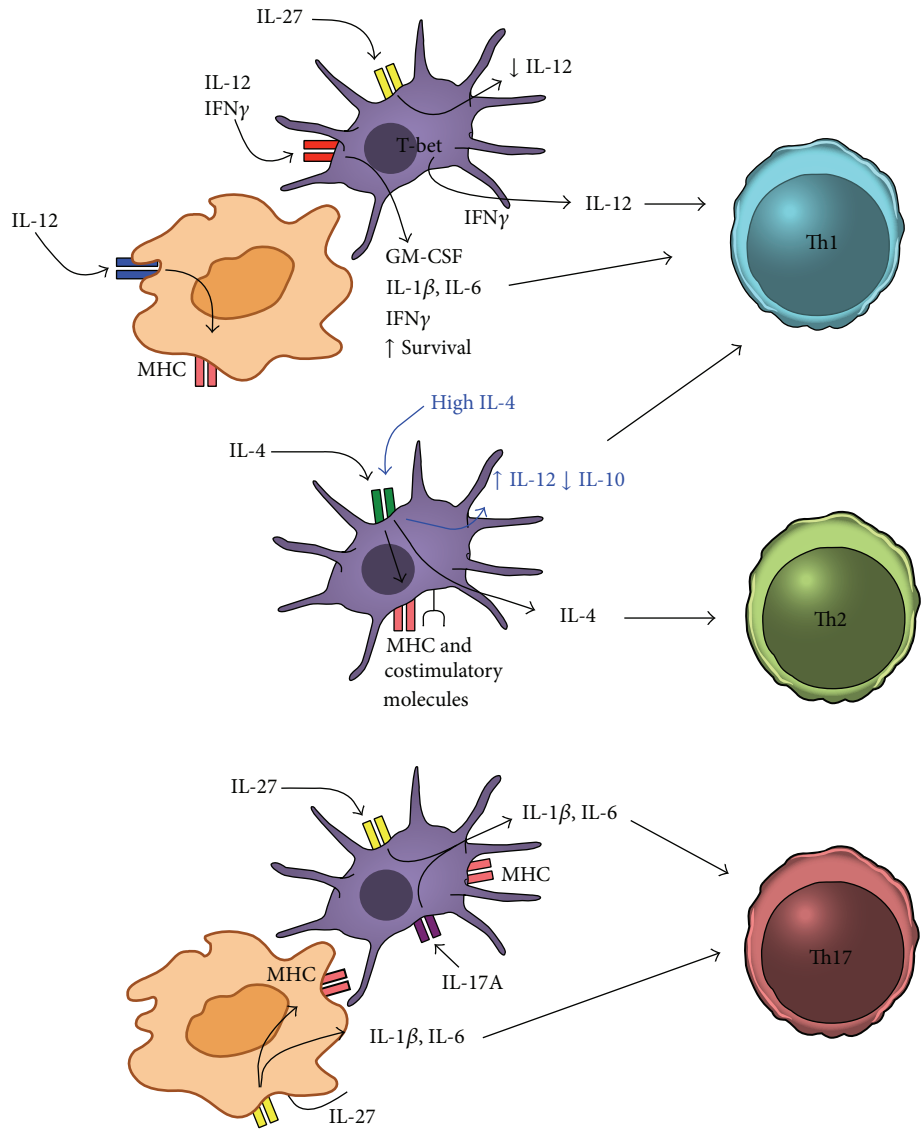

Figure 1

6. Conclusion

Activated APCs are the main sources of cytokines capable of influencing adaptive T cell responses. As discussed in this review, there is evidence that APCs are also potent target cells of these cytokines, and the stimulation of APCs might have equally important roles in generating different effector immunity (Figure 1). Therefore, many cytokines will affect T cell immunity by acting on the T cells in concert with its targeting on APCs. In order to dissect the pathways in vivo, utilizing animal models with a cell type specific deletion of cytokine receptors will be an ideal approach. Endeavor to understand the pathways has already begun.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This paper is supported by NIH Grants R01-AI074932 (B.M.) and T32-GM088088 (A.V.) and by the Crohn's & Colitis Foundation of America SRA no. 291524 (B.M.).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 589672, 7 pages
http://dx.doi.org/10.1155/2014/589672

Review Article

The Role of Protein Modifications of T-Bet in Cytokine Production and Differentiation of T Helper Cells

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Received 14 March 2014; Accepted 15 April 2014; Published 13 May 2014

Academic Editor: Mizuko Mamura

T-Bet (T-box protein expressed in T cells, also called as TBX21) was originally cloned as a key transcription factor involved in the commitment of T helper (Th) cells to the Th1 lineage. T-Bet directly activates IFN-γ gene transcription and enhances development of Th1 cells. T-Bet simultaneously modulates IL-2 and Th2 cytokines in an IFN-γ-independent manner, resulting in an attenuation of Th2 cell development. Numerous studies have demonstrated that T-bet plays multiple roles in many subtypes of immune cells, including B cell, dendritic cells, natural killer (NK) cells, NK T cells, and innate lymphoid cells. Therefore, T-bet is crucial for the development and coordination of both innate and adaptive immune responses. To fulfill these multiple roles, T-bet undergoes several posttranslational protein modifications, such as phosphorylation at tyrosine, serine, and threonine residues, and ubiquitination at lysine residues, which affect lineage commitment during Th cell differentiation. This review presents a current overview of the progress made in understanding the roles of various types of T-bet protein modifications in the regulation of cytokine production during Th cell differentiation.

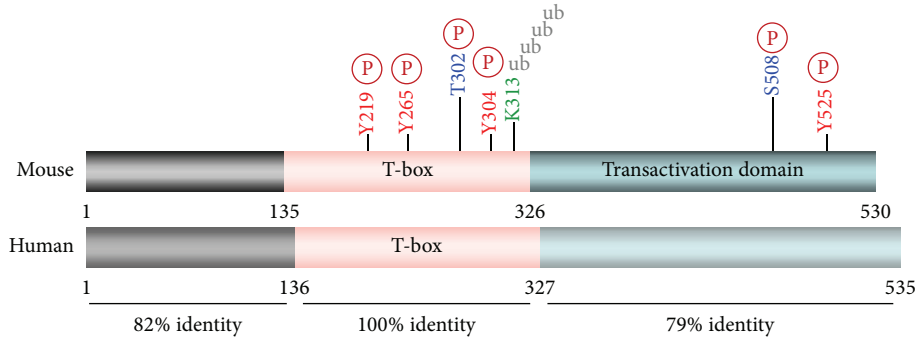
1. Introduction

T-Bet (T-box protein expressed in T cells, also called TBX21) was firstly described in 2000 in a report examining the effects of T-bet on the differentiation of T helper 1 (Th1) cells [1]. For the past 15 years, many studies have examined the functions of T-bet and have revealed multiple roles for this protein during Th cell differentiation, with a focus on the molecular mechanisms involved, the novel functions of this transcription factor in innate immune cells, and T-bet-mediated modulation of inflammatory diseases [2–9]. It has been clarified that T-bet plays a critical role in the coordination of innate immunity and adaptive immunity and that it fulfills an important function in modulating chronic inflammatory diseases, including asthma and inflammatory bowel disease, by controlling a network of highly conserved genetic programs [10–12]. Thus, optimal regulation of T-bet expression and activity seems to be beneficial for preventing or treating chronic inflammation and autoimmune diseases.

Although attempts have been made at identifying the small molecules that control the expression and activity of T-bet that affect the T cell-mediated immune response, little progress has been made on this to date. Given the importance of T-bet in the immune regulation, elucidating the functional mechanisms underlying the multiple roles of T-bet would facilitate the development of novel therapeutic interventions for treating chronic inflammatory and autoimmune diseases. This review summarizes the current state of knowledge about the molecular mechanisms underlying the multiple roles played by T-bet in Th cell development.

2. Structure of T-Bet

The T-bet contains an amino-terminus, a T-box domain, and a carboxyl-terminus, which show 82%, 100%, and 79% homology, respectively, between mice (530 amino-acid residue protein) and humans (535 amino-acid residue protein) (Figure 1). The T-box domain, located between residues 135 and 326 in mouse T-bet, is highly conserved in 18 members of the T-box protein (TBX) family [13, 14]. Common features shared by T-box proteins include a capacity for DNA binding through the T-box domain and transcriptional



Position	Modification	Physiological effect	Reference
Y219/Y265/Y304	c-Abl-mediated phosphorylation	Induction of Th1 cell development Suppression of Th2 cell development	[28]
T302	Phosphorylation	Interaction with NFAT Suppression of IL-2 and Th2 cytokines	[29]
Y304	—	Interaction with RUNX1 Inhibition of Th17 cell development	[21]
K313	Ubiquitination	Binding to DNA sequence Control of protein stability	[29]
S508	GSK-3-mediated phosphorylation	Interaction with NF-κB p65 Inhibition of IL-2	[19]
Y525	ITK-induced phosphorylation	Interaction with GATA-3 Suppression of Th2 cytokines	[5]

Figure 1: Structure and protein modification of T-bet. Mouse and human T-bet is 100% identical in the T-box domain. Several amino acid residues are conserved in mice and undergo posttranslational modifications, including phosphorylation at serine, threonine, and/or tyrosine residues, and ubiquitination at lysine residues.

regulatory activity, which plays a role in controlling the expression of developmental gene in all animal species.

The T-box domain is made up of about 180 amino-acid residues and is both sufficient and necessary for binding to the consensus DNA sequence TCACACCT [13–15]. Brachyury (T) was the first T-box protein to be identified and, in dimeric form, interacts with the major and the minor grooves of DNA through hydrophobic interactions and unusual main-chain carbonyl contact with a guanine as a dimer [16]. TBX1 also binds to the DNA sequence as a dimer, whereas TBX2 appears to bind to the same DNA sequence as a monomer [17]. Although TBX1 and TBX2 share 61% identity in the T-box domain, the structure of the DNA-T-box binding complex appears to be different, because of the low homology among the amino- and carboxyl-terminal regions. The T-box domain in T-bet shows 50% homology with the corresponding domain in brachyury (T), TBX1, and TBX2; however, the crystal structure of T-bet bound to the DNA sequence remains to be characterized.

3. Regulation of Th Cell Differentiation by T-Bet

3.1. Stimulation of Th1 Cell Differentiation by T-Bet. T-bet directly binds to the consensus DNA sequence within the *IFNG* promoter and activates its transcription. The T-bet-induced expression of *IFNG* derives Th precursor cells to differentiate into Th1 effector cells. While exogenous T-bet overexpression in naïve Th cells preferentially increases development of Th1 cells, T-bet deficiency leads to a failure to produce sufficient IFN-γ and therefore reduces generation of Th1 cells [1, 18]. T-Bet expression is substantially increased by stimulation of the T cell receptor (TCR) and is augmented by cotreatment with IFN-γ and IL-12. IFN-γ binds to its receptor and induces activation of signal transducer and activator of transcription (STAT) 1 and transcription of T-bet gene (*TBX21*). Subsequently, T-bet directly stimulates the transcription of *IFNG* as well as *IL12RB2*. Expression of IL-12 receptor (IL-12R) β2 on the cell surface further enhances IFN-γ production through IL-12 and the STAT4 signaling pathway, thereby resulting in preferential Th1 cell differentiation. Interestingly, enforced T-bet expression can also convert the differentiated Th2 cells into Th1 cells [1]. Therefore, T-bet is positioned at the crux of the regulatory pathways that induce IFN-γ in Th cells.

3.2. Attenuation of IL-2 Production by T-Bet. In addition to IFN-γ regulation, T-bet significantly suppresses IL-2 expression. This cytokine, an early T cell growth factor, is essential for activation, proliferation, and differentiation of Th cells and

is abundantly produced upon TCR stimulation. Ectopically introduced T-bet significantly suppresses IL-2 production through inhibition of nuclear factor κB (NF-κB) p65 activity, under conditions of both Th1 and Th2 differentiation [19]. During Th1 cell differentiation, IL-2 transcription is also attenuated upon induction of T-bet. The T-bet-mediated IL-2 inhibition may affect Th cell expansion and exquisitely modulate the Th1-mediated immune response upon exposure to a pathogenic antigen.

3.3. Suppression of Th2 Cell Development by T-Bet. Furthermore, exogenous T-bet introduction into Th cells suppresses the production of Th2 cytokines, such as IL-4, IL-5, and IL-13, via suppression of GATA-binding protein-3 (GATA-3). Accordingly, a lack of T-bet induces spontaneous Th2 cell development in vitro and in vivo [1, 18]. The Th2-suppressive activity of T-bet was also confirmed in the absence of IFN-γ, indicating that T-bet has a discrete inhibitory function, independent of IFN-γ stimulation.

3.4. Other Functions of T-Bet. Recently, many studies have reported that T-bet also modulates other Th cell lineages, including Th17, Treg, and follicular Th (T_{FH}) cells, in coordination with many transcription factors, such as the retinoic acid-related orphan receptor-γt (RORγt), runt-related transcription factor 3 (RUNX3), and B-cell lymphoma-6 (BCL6) [20–25]. These findings suggest that T-bet is a transcription factor that is critical for fine-tuning Th cell development.

4. Posttranslational Modification of T-Bet

T-Bet functions as a multitasking player in the regulation of Th cell differentiation. However, the molecular mechanisms that underlie the stimulatory and inhibitory activity of T-bet in regulating target gene expression remain to be clarified. Many multitasking proteins are known to undergo posttranslational protein modifications and to determine cell fates by exerting direct stimulatory and indirect inhibitory activity on target gene expression [26, 27].

4.1. Tyrosine Phosphorylation of T-Bet. Antibody-based detection of T-bet proteins in western blots results in multiple bands, suggesting the posttranslational modification of T-bet in TCR-triggered Th cells. Tyrosine phosphorylation of T-bet protein occurs primarily during the early stages (days 2 to 3) of Th cell development, upon TCR engagement, and declines afterwards. Treating Th cells with the tyrosine phosphatase inhibitor pervanadate enhances the tyrosine phosphorylation of T-bet. T-Bet is mainly localized in the nucleus, and the nuclear tyrosine kinase IL2-inducible tyrosine kinase (ITK) was identified as the responsible upstream tyrosine kinase. ITK deficiency prevents tyrosine phosphorylation of T-bet in Th cells after stimulation with TCR and IL-12. Mutational research has revealed that tyrosine residue 525 (Y525) is the relevant phosphorylation site and that phosphorylation at this site plays an important role in the interaction with GATA-3. Although T-box domain in T-bet is important for DNA and protein-protein interaction, tyrosine phosphorylation of Y525 is prerequisite for the suppression of GATA-3-mediated Th2 cell differentiation. Blockade of Y525 phosphorylation abrogates the interaction with and suppression of GATA-3, resulting in impairment of Th2 suppression [5].

Furthermore, another nuclear tyrosine kinase, c-Abl, induces phosphorylation of T-bet at tyrosine residues 219, 265, and 304 in mouse T-bet [28]. A deficiency in c-Abl as well as mutation of T-bet at these residues (Y219/265/304F mutants) leads to a failure to increase IFN-γ induction and to suppress Th2 cytokine production, due to the loss of phosphorylation at these tyrosine residues. This, in turn, results in the aggravation of allergic lung inflammation in vivo [28]. These findings suggest that ITK- and c-Abl-induced tyrosine phosphorylation of T-bet is essential for the modulation of Th2 cell development and the allergic immune response.

4.2. Serine Phosphorylation of T-Bet. Although T-bet-mediated suppression of Th2 cell development is impaired by mutation of Y525 and the absence of c-Abl kinase, IL-2 suppression is retained with a Y525-mutant T-bet, suggesting the existence of an additional regulatory mechanism for IL-2 modulation [5]. Interestingly, the appearance of multiple bands of T-bet protein on western blots could be eliminated by the addition of calf intestinal phosphatase, which predominantly eliminates phosphorylation at serine/threonine residues. Mass spectrometric analysis then revealed serine 508 (S508) as another phosphorylation site in T-bet. Mutation of S508 abolishes casein kinase- (CK-) and glycogen synthase kinase-3 (GSK-3-) mediated phosphorylation in T-bet, as well as the IL-2–suppressive activity of the protein. Moreover, S508 phosphorylation is important for the interaction of T-bet with NF-κB p65 and for prevention of binding of NF-κB p65 to the *IL2* promoter. In accordance with the function of T-bet as an NF-κB p65 inhibitor, T-bet-null Th1 cells sustain NF-κB p65 activity during Th1 cell differentiation and thus produce more IL-2. Therefore, it has been suggested that T-bet is a physiological inhibitor of IL-2 during Th1 cell differentiation, through S508 phosphorylation-dependent suppression of NF-κB p65.

4.3. Threonine Phosphorylation of T-Bet. Very recently, threonine 302 (T302) was characterized as a novel phosphorylation site in T-bet [29], although it remains unclear which kinase and phosphatase affect the phosphorylation of this residue. However, restoration of T-bet-null Th cells with a T302-mutant T-bet stimulated IFN-γ production as much as did wild-type T-bet; however, the mutant failed to suppress IL-2 and other Th2 cytokines. Further analysis demonstrated that T302 phosphorylation is required for the interaction of T-bet with nuclear factor of activated T cells (NFAT) and for down-regulation of NFAT-mediated IL-2 and Th2 cytokines, such as IL-4, IL-5, and IL-13. NFAT is not crucial for induction of IFN-γ production and T302-mutant T-bet is able to bind to the *IFNG* promoter; thus, IFN-γ production was comparable between wild-type and mutant T-bet. In other words, mutation of T302 abrogated the T-bet-mediated suppression of IL-2 and Th2 cytokine production but did not affect the DNA-binding and IFN-γ-stimulatory activity of T-bet.

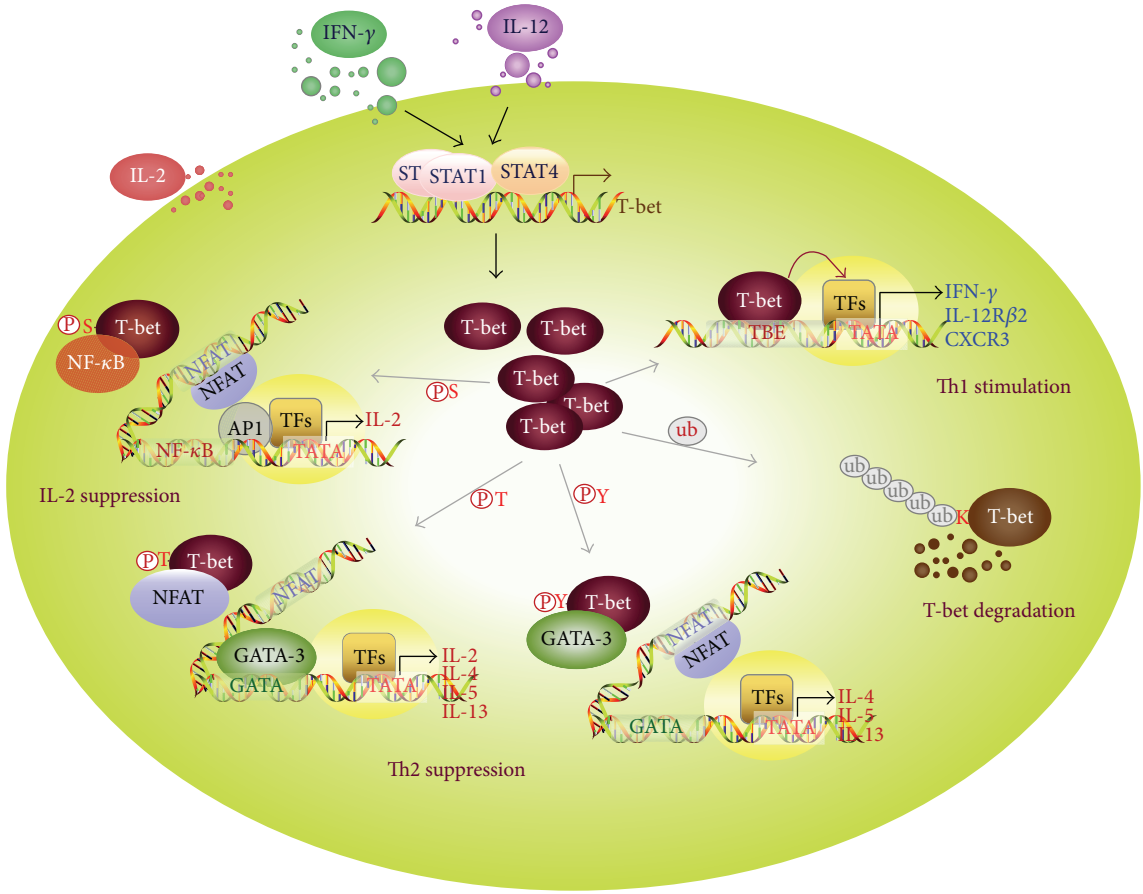


Figure 2: Multiple T-bet functions playing a role in Th cell differentiation. Induction of T-bet expression through activation of STAT1 and STAT4 directly stimulates the transcription of T-box-binding element-containing genes, such as *IFNG*, *IL12RB2*, and *CXCR3*, thereby enhancing Th1 cell development. T-Bet undergoes serine phosphorylation at S508 and then downregulates IL-2 production in Th1 cells by recruiting NF-κB p65 from the *IL2* promoter. Protein levels of T-bet in Th1 cells can be controlled by the ubiquitin-mediated proteasomal degradation pathway. Moreover, T-bet protein undergoes additional posttranslational modifications, for example, phosphorylation at T302 and Y525, which facilitates its suppression of the Th2 cytokine production that is induced by activation of NFAT and GATA-3.

Indeed, T302 is located in the DNA-binding T-box, as is Y304 [28]. The T-box domain consists of several repeats of β-strands and α-helices and is involved in both dimerization and DNA binding [16]. Muller and Herrmann predicted that the α-helices αH3 and αH4 in brachyury (T) are important for the direct interaction of this protein with the minor and major grooves of DNA [16]. However, T302 may not be associated directly with the DNA grooves, regardless of its phosphorylation status. It would be interesting to know which upstream kinase and phosphatase regulates T302 phosphorylation and whether T302 phosphorylation affects other protein modifications of T-bet.

4.4. Ubiquitination of Lysine 313 in T-Bet. T-bet expression is critical for the transcriptional regulation of *IFNG* and for the development of Th1 cells, but the means of regulation of T-bet at the protein level is yet to be identified. Jang et al. have recently reported that T-bet undergoes ubiquitination-mediated proteasomal degradation during the later stages of Th1 cell differentiation [29]. Of the 16 lysine residues present in mouse T-bet protein, 11 are predominantly located within the T-box domain, and the remaining 5 are located at the carboxyl-terminus (residues 326 through 530), while no lysine residues are present in the amino-terminus (residues 1 through 134). Interestingly, lysine residues within the T-box domain are preferentially ubiquitinated upon overexpression of ubiquitin. Further analysis has identified that mutation of lysine 313 (K313) decreases ubiquitination-mediated T-bet degradation and enhances the expression level of T-bet in the nucleus and the cytoplasm. Despite the increased levels of the K313 mutant, this mutation completely abrogated T-bet functions involving DNA binding, transcriptional activation of *IFNG*, and suppression of IL-2 and Th2 cytokine production. The crystal structure of the α-helices of the T-box domain bound to DNA strongly suggests that the amino group of K313 is associated with the phosphate of a DNA base via hydrogen-bond interaction. In addition, mutation of K313 also leads to failure to suppress IL-2 and Th2 cytokine production; however, the interaction with and suppression of GATA-3 and NF-κB p65 are not altered by mutation of K313. Interestingly, NFAT interaction is abolished in K313-mutant T-bet, which is also strongly associated with an absence of

phosphorylation at T302. It is not clear yet whether and how K313 regulates T302 phosphorylation and vice versa.

5. T-Bet in Inflammatory and Autoimmune Diseases

Since Mosmann et al. discovered Th1 and Th2 subsets that produce signature cytokines, IFN-γ, and IL-4, IL-5, and IL-13, respectively, and that modulate inflammatory and allergic immune responses [30], further studies have identified novel subsets of Th cells, such as Th17, T_{FH}, and Treg cells [11, 21, 23, 24, 31–34]. Extensive studies have also characterized the cytokine signaling pathways and transcription factors involved in the regulation of immune responses to pathogens [1, 35–54]. Importantly, T-bet plays a fundamental role in controlling differentiation of several subsets of Th cells and in modulating inflammatory and autoimmune diseases [10–12].

T-Bet also functions as an antiasthmatic regulator. A deficiency in T-bet spontaneously leads to the development of asthmatic symptoms, which is characterized by increased eosinophil infiltration into the airway, mucus-secreting goblet cell hyperplasia, and chronic airway remodeling with collagen accumulation and proliferative myofibroblasts; these features are often also seen in asthmatic patients [55–57]. Restoration of T-bet expression shifts the immune balance to a Th1 response and prevents and attenuates pathologic lung inflammation in vivo [58, 59].

Moreover, T-bet is protected against intracellular pathogenic infections. Abrogation of T-bet-induced IFN-γ production resulted in higher susceptibility to intracellular pathogens in vivo, including *Mycobacterium tuberculosis*, *Leishmania major*, and *Salmonella typhimurium* [18, 60, 61], emphasizing the importance of IFN-γ production by T-bet-expressing Th1 cells in the defense against bacterial infections. However, T-bet-deficient mice are resistant to infection by *Listeria monocytogenes*, because INF-γ production by natural killer cells is both necessary and sufficient for the host defense against *L. monocytogenes* [62].

Furthermore, T-bet can aggravate the development of inflammatory and autoimmune diseases, including inflammatory bowel disease, experimental autoimmune encephalomyelitis, inflammatory arthritis, and type I diabetes, as these inflammatory diseases are attenuated in the absence of T-bet [6, 56, 63, 64]. These findings suggest that fine-tuning of the immune response by modulation of T-bet expression could have beneficial effects for patients with chronic asthma, inflammatory bowel disease, arthritis, multiple sclerosis, and diabetes.

6. Conclusions and Perspectives

T-bet is a T-box domain-containing transcription factor that is typically involved in developmental regulation but exerts multiple functions in Th cell differentiation; transcriptional activation of IFN-γ-expressing Th1 cells, indirect suppression of Th2, Th17, and Treg cell development, and fine-modulation of IL-2 production in Th1 cells. This multitasking is not surprising, as T-bet undergoes several posttranslational modifications. Phosphorylation at Y525 plays a role in GATA-3 suppression during Th2 regulation, phosphorylation at S508 causes NF-κB p65 suppression in the context of IL-2 regulation in Th1 cells, phosphorylation at T302 plays a role in fine-tuning IL-2 production, and ubiquitination at K313 plays a role in controlling T-bet protein stability. Thus, posttranslational modification of T-bet facilitates its functional diversity and the complexity of its modulation of cytokine expression (Figure 2). It is not known whether the various posttranslational modifications occur sequentially or simultaneously, whether one type of protein modification affects other modification, or whether changes in the posttranslational modification of T-bet are related to the development of infectious and chronic inflammatory diseases. Further identification of novel protein modifications related to T-bet functions would provide valuable insights into the development of powerful therapeutic interventions for controlling chronic inflammatory and autoimmune diseases.

List of Abbreviations

BCL-6: B-cell lymphoma-6
CK: Casein kinase
GATA-3: GATA-binding protein 3
GSK-3: Glycogen synthase kinase-3
IFN: Interferon
IL: Interleukin
ITK: IL-2-inducible T cell kinase
NFAT: Nuclear factor of activated T cells
NF-κB: Nuclear factor kappa B
RORγt: Retinoic acid-related orphan receptor gamma t
RUNX: Runt-related transcription factor
STAT: Signal transducer and activator of transcription
T-bet: T-Box protein expressed in T cells
Th: T helper
Treg: Regulatory T
T_{FH}: Follicular T helper
Ub: Ubiquitin.

Conflict of Interests

No potential conflict of interests was disclosed.

Acknowledgment

This work was supported by Mid-Career Researcher Program through NRF Grant (2013R1A2A2A01068302).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 149185, 19 pages
http://dx.doi.org/10.1155/2014/149185

Review Article

Chronic Inflammation and Cytokines in the Tumor Microenvironment

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Received 10 February 2014; Accepted 15 April 2014; Published 13 May 2014

Academic Editor: Evelin Grage-Griebenow

Acute inflammation is a response to an alteration induced by a pathogen or a physical or chemical insult, which functions to eliminate the source of the damage and restore homeostasis to the affected tissue. However, chronic inflammation triggers cellular events that can promote malignant transformation of cells and carcinogenesis. Several inflammatory mediators, such as TNF-α, IL-6, TGF-β, and IL-10, have been shown to participate in both the initiation and progression of cancer. In this review, we explore the role of these cytokines in important events of carcinogenesis, such as their capacity to generate reactive oxygen and nitrogen species, their potential mutagenic effect, and their involvement in mechanisms for epithelial mesenchymal transition, angiogenesis, and metastasis. Finally, we will provide an in-depth analysis of the participation of these cytokines in two types of cancer attributable to chronic inflammatory disease: colitis-associated colorectal cancer and cholangiocarcinoma.

1. Introduction

The role of inflammation in the development of cancer was described as early as 1863, by Rudolf Virchow. His observations that inflammatory cells infiltrate tumors led him to hypothesize that cancer arises from inflammatory sites ("lymphoreticular infiltration") [1, 2]. In the last decades, Virchow's postulation has been supported by abundant evidence that various cancers are triggered by infection and chronic inflammatory disease [3].

Inflammation is a beneficial response activated to restore tissue injury and pathogenic agents. However, if inflammation is unregulated, it can become chronic, inducing malignant cell transformation in the surrounding tissue. The inflammatory response shares various molecular targets and signaling pathways with the carcinogenic process, such as apoptosis, increased proliferation rate, and angiogenesis. Furthermore, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to decrease incidence and mortality of several cancers [4].

In relation to chronic inflammatory-associated neoplasias, this review article explores the involvement of cytokines in chronic inflammation and carcinogenesis, focusing on inflammatory bowel disease-associated cancer and cholangiocarcinoma (CCA) induced by chronic inflammation of biliary ducts, that is, primary sclerosing cholangitis (PSC) and liver fluke associated-CCA. Both cancers are examples of a localized, long-term inflammatory process increasing the risk of cancer.

2. Chronic Inflammation as an Inducer of Tumors

The immune response comprises a series of events triggered in response to recognition of pathogens or tissue damage, involving cells and soluble mediators, such as cytokines of

the innate and adaptive immune system. The main purpose of this inflammatory response is to remove the foreign agent disturbing tissue homeostasis [5]. In the normal physiological context, after tissue repair or pathogen elimination, the inflammation is resolved and the homeostatic state recovered [6].

It is now widely accepted that inadequately resolved chronic inflammation may increase the risk of cancer. Several pathologies illustrate this link, such as endometriosis, chronic prostatitis, and chronic gastritis due to *Helicobacter pylori* (*H. pylori*), inflammatory bowel diseases (IBD), and primary sclerosing cholangitis (PSC) (Table 1). Inflammation can increase the risk of cancer by providing bioactive molecules from cells infiltrating the tumor microenvironment, including cytokines; growth factors; chemokines that maintain a sustained proliferative rate; cell survival signals to avoid apoptosis; proangiogenic factors; and extracellular matrix-modifying enzymes such as metalloproteinases that promote epithelial-mesenchymal transition (EMT) and facilitate other carcinogenesis programs, such as genome instability, reprogramming of energy metabolism, and immune evasion [7]. Here, we focus on key cytokines involved in tumor induction and their role in EMT, angiogenesis, invasion, and metastasis.

3. Cytokines Involved in Tumor Development

Cytokines are low-molecular-weight proteins that mediate cell-to-cell communication. Immune and stromal cells, such as fibroblasts and endothelial cells, synthesize them and they regulate proliferation, cell survival, differentiation, immune cell activation, cell migration, and death. Depending on the tumor microenvironment, cytokines can modulate an antitumoral response, but during chronic inflammation, they can also induce cell transformation and malignancy, conditional on the balance of pro- and anti-inflammatory cytokines, their relative concentrations, cytokine receptor expression content, and the activation state of surrounding cells [50].

3.1. Tumor Necrosis Factor (TNF-α). As noted, unresolved inflammation can lead to malignancy. Tumor necrosis factor (TNF-α) is one inflammatory mediator that has been implicated in carcinogenesis, due to its participation in chronic inflammatory diseases [51]. Moore et al. provided evidence that TNF-α-deficient mice are resistant to tetradecanoylphorbol-13-acetate- (TPA-) induced skin carcinogenesis. TNF-α effect seems to be more significant in the early stages of carcinogenesis, including angiogenesis and invasion, versus progression of carcinogenesis [52, 53]. While TNF-α is a prototypical proinflammatory cytokine, evidence suggests a double-edged role in carcinogenesis. This cytokine is recognized by two receptors: TNF-α receptor-1 (TNF-αR-1), ubiquitously expressed, and TNF-αR-2, expressed mainly in immune cells [54]. Trimerization occurs upon TNF-α binding to TNF-α-Rs, leading to activation of at least four signaling pathways: a proapoptotic pathway induced by caspase-8 interaction with Fas-associated death domain (FADD); an antiapoptotic platform activated by cellular inhibitor of apoptosis protein-1 (cIAP-1) and interacting with TNF-αR-associated factor 2 (TRAF2); a TRAF2- and JNK-mediated AP-1 signaling pathway; and a receptor interacting protein-(RIP-) induced NF-κB [54].

There is controversy, however, regarding the role of TNF-α in cancer; high concentrations of this cytokine can induce an antitumoral response in a murine model of sarcoma [55]. Furthermore, William B. Coley, a pioneer surgeon in the field, discovered that there was a reliable treatment response for systemic bacterial filtrate injection in sarcoma patients [55, 56]. However, severe toxic side effects have been associated with systemically administered TNF-α, such as hypotension and organ failure [57]. Local administration has been shown to be safer and effective, as demonstrated by clinical trials evaluating a TNF-α-expressing adenovirus (TNFerade) gene therapy combined with chemotherapy [58, 59]. Moreover, TNF-α-conjugate targeting peptides or single-chain antibody fragments have also shown variable effects, depending on the patient [60].

In contrast, low, sustained TNF-α production levels can induce a tumor phenotype [61]. A TNF-α tumor promotion mechanism is based on reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation, which can induce DNA damage, hence facilitating tumorigenesis [62, 63]. TNF-α-mediated inflammation has been linked to cancer; for instance, increased TNF-α levels in preneoplastic lesions have been detected in *H. pylori*-positive gastric lesions, through *H. pylori*-secreted TNF-α-inducing protein (Tipα) [64, 65].

A study by Kwong et al. explored TNF-α-associated tumorigenesis using an organoid of normal human ovarian epithelial cells exposed to a prolonged TNF-α dose. The model demonstrated generation of a precancerous-like phenotype with structural and functional changes, such as tissue disorganization, epithelial polarity loss, cell invasion, and overexpression of cancer markers [66].

According to these findings, the pro- or antitumoral TNF-α response within the tumor microenvironment depends not only on local concentration but also on its expression site in the tumor. Patients with elevated levels of TNF-α in tumor islets from non-small cell lung cancer, mainly restricted to macrophages and mast cells, showed the highest survival rates, while patients with increased stromal TNF-α content showed lower survival rates [67].

There is also evidence that prolonged TNF-α exposure can enhance the proportion of cancer stem cell phenotypes in oral squamous cell carcinoma, increasing their tumor-forming sphere ability, stem cell-transcription factor expression, and tumorigenicity [68].

3.2. Interleukin 6 (IL-6). Another proinflammatory cytokine with a typical protumorigenic effect is IL-6. Elevated serum IL-6 levels have been detected in patients with systemic cancers as compared to healthy controls or patients with benign diseases. IL-6 has been proposed as a malignancy predictor, with sensitivity and specificity of about 60–70% and 58–90%, respectively [69]. However, there are limited studies available that might be used to define cut-off values for IL-6 as a diagnostic tool.

IL-6 plays a key role in promoting proliferation and inhibition of apoptosis, by binding to its receptor (IL-6Rα)

Table 1: Cancer associated with chronic inflammatory disorders.

Cancer	Associated inflammatory stimuli	Reference
Colorectal cancer/colitis-associated cancer	Inflammatory bowel diseases (ulcerative colitis and Crohn's diseases)	[8]
Cholangiocarcinoma	Liver fluke and primary sclerosing cholangitis	[9]
Gastric cancer	Chronic gastritis (*H. Pylori*)	[10]
Lung cancer	Inflammation caused by asbestos, infections, smoking, and silica	[11]
Prostate cancer	*E. coli* infection of prostate	[12]
Hepatocellular carcinoma	Infection with hepatitis virus B and hepatitis virus C	[13]
Melanoma	UV irradiation-associated skin inflammation	[14]
Endometrial carcinoma	Endometriosis	[15]
Gall bladder carcinoma	Gall bladder stone-associated chronic cholecystitis	[16, 17]
Esophageal cancer	Barrett's esophagitis	[18]

and coreceptor gp130 (glycoprotein 130), thus activating the JAK/STAT signaling pathway of the Janus kinases (JAK) and signal transducers and activators of transcription (STATs) STAT1 and STAT3 [70]. STATs belong to a family of transcription factors closely associated with the tumorigenic processes. Several studies have highlighted the effect of the IL-6/JAK/STAT signaling pathway on cancer initiation and progression. IL-6 can induce tumorigenesis by hypermethylation of tumor suppressor genes as well as by hypomethylation of retrotransposon long interspersed nuclear element-1 (LINE-1) in oral squamous cell cancer lines *in vitro* [71], a frequent event in various human cancers. Furthermore, IL-6 has been shown to be produced primarily by stromal fibroblasts in a gastric cancer mouse model; however, the deficient mouse model exhibits reduced tumorigenesis when exposed to the carcinogen N-methyl-N-nitrosourea [72].

IL-6 has a role in multiple myeloma development, as demonstrated by its ability to induce apoptosis by blocking the IL-6R/STAT3 pathway *in vitro* [73] and the resistance of IL-$6^{-/-}$ mice to plasmacytoma induction [74].

Like TNF-α, IL-6 facilitates tumor development by promoting conversion of noncancer cells into tumor stem cells. In particular, IL-6 secretion by noncancer stem cells in low-attachment culture conditions upregulates Oct4 gene expression by activating the IL-6R/JAK/STAT3 signaling pathway [75].

These findings have led researchers to propose IL-6 as a therapeutic target in cancer. Several phase I/II clinical trials are currently evaluating antibodies against IL-6 or IL-6R as therapeutic alternatives. Siltuximab (CNTO 328), a monoclonal antibody against IL-6, has shown promising results for non-small cell lung cancer, ovarian cancer, prostate cancer, and multiple myeloma, among others [76–80].

In this context, as inflammatory cytokines are partially responsible for tumor induction, an increase in anti-inflammatory cytokines should limit the risk of cancer and reduce activation of signaling pathways. Nonetheless, evidence suggests that anti-inflammatory cytokines, such as TGF-β and IL-10, show more complex effects on tumor development.

3.3. Transforming Growth Factor β (TGF-β). TGF-β is a powerful pleiotropic cytokine, with immune-suppressing and anti-inflammatory properties. Under physiological conditions, TGF-β has a well-documented role in embryogenesis, cell proliferation, differentiation, apoptosis, adhesion, and invasion [81]. Three isoforms have been identified: TGF-β1, TGF-β2, and TGF-β3. TGF-βs binds to the cognate type II receptor (TGF-β RII), inducing type I TGF-β receptor (TGF-β RI) phosphorylation and leading to the formation of a heterotetrameric complex that activates SMAD-dependent transcription [82]. SMAD transcription factors are structurally formed by a serine and threonine-rich linker region that connects two MAD (mothers against dpp) homology regions. Differential phosphorylation of these amino acid residues contributes to various cellular functions, including cytostatic effects, cell growth, invasion, extracellular matrix synthesis, cell cycle arrest, and migration [83]. Therefore, differential phosphorylation of SMAD2 and SMAD3 by TGF-β receptor activation promotes their translocation into the nucleus, where they form a complex with SMAD4, further bind to DNA, associate with other transcription factors, and induce gene expression [82].

The role of TGF-β in cancer is complex and paradoxical, varying by cell type and stage of tumorigenesis. In early stages, TGF-β acts as a tumor suppressor, inhibiting cell cycle progression and promoting apoptosis. Later, TGF-β enhances invasion and metastasis by inducing epithelial-mesenchymal transition (EMT) [84]. In cancer induction, TGF-β exerts a tumor suppressor effect through cyclin-dependent kinase inhibitor (CKI) p21 upregulation and c-Myc downregulation [85]. Using a conditional TGF-β RII knock-out mice model, Guasch et al. found that highly proliferative epithelia (such as rectal and genital) developed spontaneous squamous cell carcinomas and furthermore showed accelerated carcinoma progression, Ras mutations, and apoptosis reduction [86], suggesting that a deficient TGF-β pathway contributes to tumorigenesis.

There is consistent evidence demonstrating that TGF-β signaling changes are involved in human cancer. Increased TGF-β1 mRNA and protein have been observed in gastric carcinoma, non-small cell lung cancer, and colorectal and prostate cancer [87], and TGF-β receptor deletion or mutations have been associated with colorectal, prostate, breast, and bladder cancer, correlating with a more invasive and advanced carcinoma, higher degree of invasion, and worse prognosis [88].

In the tumor microenvironment, common sources of TGF-β are cancer and stromal cells, including immune cells and fibroblasts [82]. Bone matrix is also an abundant source of TGF-β and a common site for metastasis in many cancers, correlating with the tumor-promoting and invasive effects of this cytokine [89].

Specific therapy targeting this cytokine in advanced cancer patients has shown promising results in preclinical and clinical studies, using TGF-β inhibitors, specifically ligand traps, antisense oligonucleotides, receptor kinase inhibitors, and peptide aptamers. Nevertheless, serious side effects of systemic TGF-β inhibitors administration have been reported, indicating that further clinical trials are required to evaluate localized, safe, dose-effective therapies [89].

3.4. Interleukin 10 (IL-10). Interleukin 10 (IL-10) is known to be a potent anti-inflammatory cytokine. Almost all immune cells, including T cells, B cells, monocytes, macrophages, mast cells, granulocytes, dendritic cells, and keratinocytes, produce IL-10 [90]. Tumor cells can also secrete IL-10, as can tumor-infiltrating macrophages [91, 92].

When IL-10 binds to its receptor, Jak1 and Tyk2 tyrosine kinases phosphorylate an IL-10R intracellular domain, allowing it to interact with STAT1, STAT3, and STAT5, favoring STAT translocation into the nucleus and induction of target gene expression [93].

Several studies have indicated that IL-10 has both pro- and antitumoral effects. IL-10 inhibits NF-κB signaling; therefore, it can downregulate proinflammatory cytokine expression [94] and act as an antitumoral cytokine. Consistent with this finding, Berg et al. demonstrated that IL-10-deficient murine models are prone to bacteria-induced carcinogenesis [95], whereas the adoptive transfer of IL-10-expressing $CD4^+CD25^+$ T cells into $Rag2^{-/-}$ (lymphocyte-deficient) mice inhibits colorectal inflammation and carcinomas [96, 97]. Moreover, IL-10 can exert antitumoral activity in gliomas, melanomas, and breast and ovarian carcinomas [98], through a mechanism involving MHC-I downregulation, thus inducing NK-mediated tumor cell lysis [99].

Due to its immunosuppressive effect on dendritic cells and macrophages, IL-10 can dampen antigen presentation, cell maturation, and differentiation, allowing tumor cells to evade immune surveillance mechanisms [100].

In addition and as previously described for IL-6, STAT3 can also be activated by IL-10, although the cytokines' contradictory responses are determined by receptor and time frame of STAT activation. In particular, IL-6 leads to a transient, rapidly declining STAT3 phosphorylation and nuclear localization, whereas IL-10 induces a sustained STAT3 phosphorylation [101]. Through STAT3 activation, IL-10 can also have a protumorigenic effect, mediated by an autocrine-paracrine loop [102] involving Bcl-2 upregulation and apoptosis resistance activation [103, 104]. Likewise, elevated IL-10 levels are associated with poor prognosis in diffuse B cell lymphoma [105] and expression by tumor cells, and tumor-associated macrophages promote Burkitt's lymphoma through the increased production of a TNF-α family member, BAFF, a tumor growth/survival molecule [106].

4. Inflammatory Response and Malignancy

4.1. Inflammation-Induced Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) in the Carcinogenic Process. In an inflammatory response, epithelial and immune cell activation trigger ROS and RNS generation through induction of NADPH oxidase and nitric oxide synthase (NOS), respectively. NADPH oxidase is a protein complex composed of several membrane-associated subunits that catalyze the superoxide anion ($O_2^{-\bullet}$), leading to superoxide dismutase-(SOD-) mediated hydrogen peroxide (H_2O_2) production. NADPH oxidase is expressed in phagocytic and nonphagocytic cells, and cytochrome subunit isoforms are present in different cell types (NOX2 in phagocytic cells, such as macrophages and neutrophils) (NOX1, 3–5, and DUOX1, 2 in nonphagocytic cells) [107]. On the other hand, NOS generates nitric oxide (NO) from L-arginine, which can be converted into RNS such as nitrogen dioxide ($NO_2^{\bullet}$), peroxynitrite ($ONOO^-$), and dinitrogen trioxide (N_2O_3). Different NOS isoforms are produced depending on cell type: inducible NOS (iNOS) in phagocytic cells and constitutive in endothelial and neuronal (eNOS and nNOS) cells [108]. ROS and RNS have a potent antimicrobial role in phagocytic cells and also act as a second messenger in signaling transduction [109, 110].

Phagocytic cell activation can directly induce reactive oxygen and nitrogen species (collectively called RONS), activating NOX2, NADPH oxidase, and iNOS [109]. Furthermore, TNF-α, IL-6, and TGF-β trigger RONS generation in nonphagocytic cells [111–113].

Increased expression of NADPH oxidase and NOS and their products RONS has been identified in various cancers, suggesting that free radicals have a role in genesis and malignant progression [63]. In various chronic inflammatory diseases, such as *H. pylori*-associated gastritis and inflammatory bowel diseases (IBD), high RONS levels have been observed, suggesting a role in cancer risk [114–116].

Different mechanisms have been proposed to clarify RONS participation in cancer development. RONS induce cell oxidative stress and damage of lipids, proteins, and DNA, as well as production of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG), which is actually used as a DNA damage marker. Furthermore, 8-oxodG can pair with adenine, leading to transversion of G:C to T:A ($G \rightarrow T$ transversion). Similarly, $ONOO^-$ can modify deoxyguanosine to 8-nitrodeoxyguanosine, which can spontaneously generate an apurinic site, favoring $G \rightarrow T$ transversion [19]. Identification of these DNA damage markers in chronic inflammatory processes, such as *H. pylori*-associated gastritis, hepatitis, and ulcerative colitis, emphasizes the relevance of RONS in pathologies with an increased risk of cancer (Figures 1(a) and 1(b)) [19, 117, 118]. Moreover, 8-oxodG and 8-nitrodeoxyguanine immune-reactivity is increased in the liver of hepatitis C virus-derived chronic hepatitis patients [118].

Jaiswal et al. found increased iNOS, 3-nitrotyrosine, and 8-oxodG in the livers of primary sclerosis cholangitis (PSC) patients [119]. Furthermore, RNS interfere with DNA repair, as shown in cells overexpressing iNOS that are unable to repair modified 8-oxodG [119]. Deficient DNA-repair

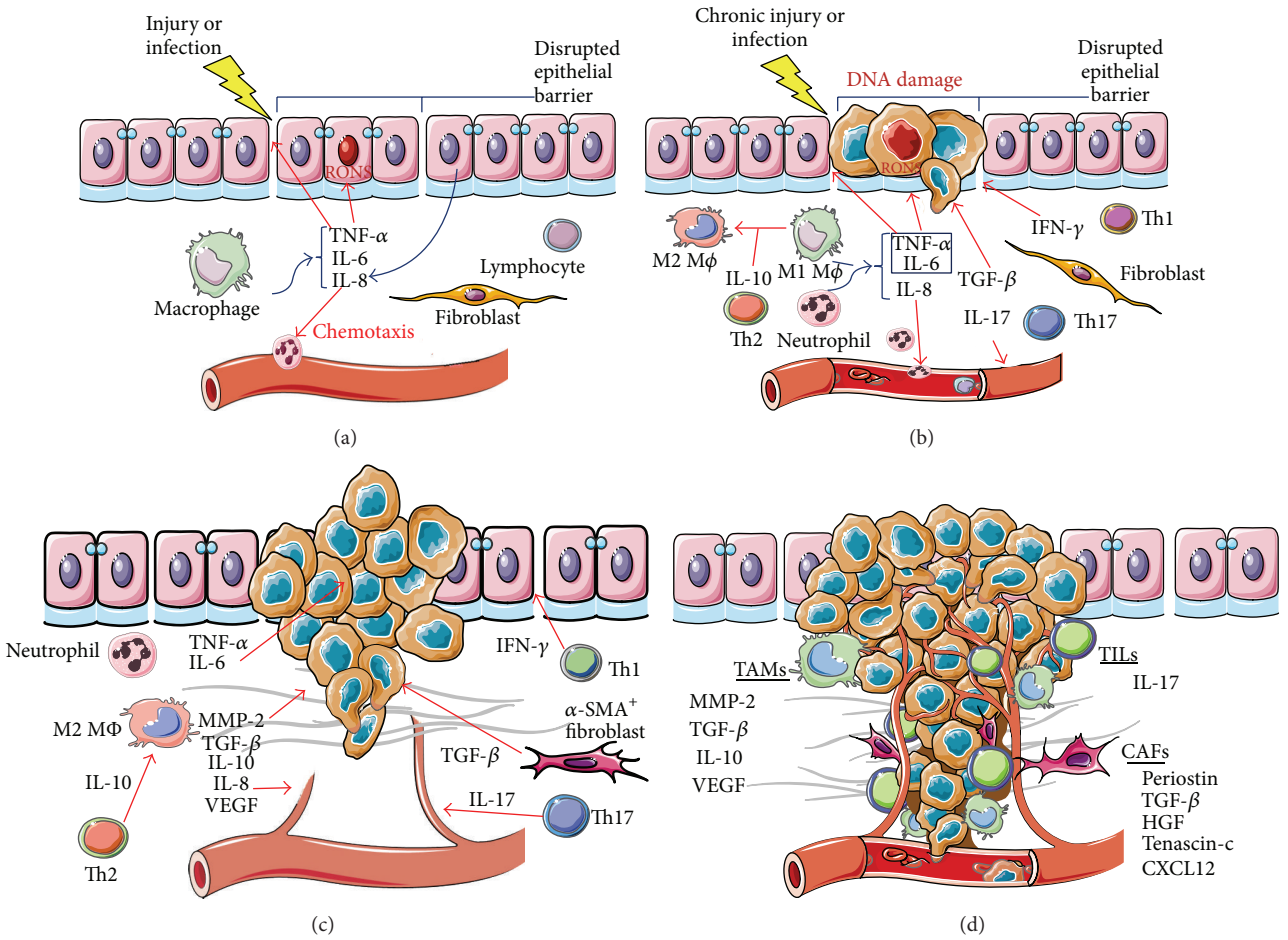

Figure 1: Schematic illustration of the role of cytokines in carcinogenesis. (a) During tissue injury or infection, an immune response activates the expression of proinflammatory mediators, such as TNF-α, IL-6, and IL-8 from macrophages and neutrophils. These cytokines can disrupt the epithelial barrier, induce RONS, and promote the infiltration of other inflammatory cells. (b) In chronic inflammation, proinflammatory cytokines such as TNF-α can induce DNA damage through RONS, which leads to tumor initiation. TGF-β can promote malignant transformation through EMT activation. Cytokines derived from $CD4^+$lymphocytes, such as IFN-γ, IL-10, and IL-17, can participate in epithelial barrier disruption, M2 phenotypic transitions of macrophages, and angiogenesis, respectively. (c) Tumor growth and invasion are also favored by proinflammatory cytokines that stimulate cell proliferation, reduce apoptosis, and enhance EMT and angiogenesis; the latter is facilitated by VEGF and IL-8. Anti-inflammatory cytokines, such as IL-10 and TGF-β, contribute to tumor immune evasion. (d) Tumor-associated macrophages (TAM), tumor-infiltrating lymphocytes (TIL), and cancer-associated fibroblasts (CAF) secrete several factors that contribute to tumor growth and metastasis, while maintaining the immunosuppressive milieu.

protein activity has been linked to enzyme S-nitrosylation, attributable to increased RNS [120].

RONS are generated by cellular stress and macromolecule modification, although they are also involved in the regulation of signaling pathways, such as survival and cell proliferation through Akt, Erk1/2, and hypoxia-inducible factor-1 (HIF-1) activation [121, 122].

There is strong evidence linking carcinogenesis to inflammatory response and RONS, and therapeutic strategies for cancer prevention using free radicals and proinflammatory signaling inhibitors have been evaluated in animal models [123–125].

4.2. Inflammation-Associated Tumor Growth. Nowadays, it is accepted that chronic inflammation is important in generating malignancy through the exposure of proinflammatory cytokines and sustained activation of signaling pathways such as NF-κB and STAT3. Following cell transformation to a malignant state, these cytokines are also involved in tumor growth, by stimulating the proliferation of tumor cells and by evading immunosurveillance (Figures 1(b) and 1(c)).

Several cytokines have growth factor activity; a relevant cytokine is TNF-α. In a study by Zhu et al., they showed that the silencing of TNF-α in a gallbladder cell line decreases cell proliferation and invasion by an autocrine effect, affecting the activation of TNF-α/NF-κB/AKT/Bcl-2 pathway in these cells [126]. This is consistent with data previously observed by Luo et al. who revealed that NF-κB signaling is required to promote tumor cell proliferation in response to an inflammatory stimulus, and by inhibiting this transcription factor, an antitumor signal led by TNF-α/TRAIL is triggered [20]. However, in a mouse model of ovarian cancer, TNF-α can

also stimulate the secretion of other cytokines like IL-17 by $CD4^+$ T cells and promote tumor growth indirectly [127].

The protumorigenic role of IL-17 has also been implicated in other types of cancer. In mice with carcinogen-induced skin tumors, those deficient in IL-17 receptor showed a lower tumor incidence and a diminished tumor size [128].

IL-6 is another typical proinflammatory cytokine with tumor growth effect, mainly by activating JAK tyrosine kinases and the transcription factor STAT3, as seen in lung, kidney, and breast cancer in which a high expression of STAT3 has been identified [70]. Also, in cell lines of malignant fibrous histiocytoma, a high secretion of IL-6 and constitutive activation of STAT3 were reported, reflecting an increase of tumor cell proliferation [129].

In cancer, other molecules that may influence tumor growth by regulating the IL-6/STAT3 signaling pathway have been reported. Inflammatory mediators like Hmgb1, IL-23, and IL17 can promote tumor growth by activating IL-6/STAT3 pathway in a mouse model of melanoma [130]. In cholangiocarcinoma, a high expression of the tumor suppressor gene regulator, gankyrin, favors tumor proliferation, invasion, and metastasis through activation of IL-6/STAT3 signaling pathway [131]. Furthermore, embelin, a derivative from *Embelia ribes*, is known to inhibit XIAP (X-linked inhibitor of apoptosis protein) and is able to impair tumor proliferation by interfering in IL-6/STAT3 signaling [132].

Finally, the anti-inflammatory cytokine IL-10 may also contribute to tumor growth. In a mouse model of melanoma, tumors overexpressing IL-10 present a higher tumor growth mediated by an increase in tumor cell proliferation, angiogenesis, and immune evasion [133].

4.3. Inflammation-Associated Epithelial Mesenchymal Transition. The epithelial mesenchymal transition (EMT) is an important process of cellular reprogramming during embryogenesis and pathological events such as inflammation, wound healing, and cancer [134, 135]. During EMT, epithelial cells exhibit morphological changes, acquiring fibroblast characteristics. In this process, structures involved in epithelial cell-cell interaction, such as tight junctions, adherens junctions, desmosomes, and gap junctions, are lost, and the cells undergo actin cytoskeleton reorganization and changes in the expression profile of proteins allowing for cell-cell contact, such as E-cadherin. Furthermore, expression of fibroblast markers, including fibronectin, α-smooth muscle actin (α-SMA), and matrix metalloproteinases, is favored during EMT. Cellular reprogramming is orchestrated by a variety of transcription factors, such as Snail, ZEB, and the helix-loop-helix (HLH) family [136, 137]. The mesenchymal phenotype provides increased motility that is associated with invasiveness and metastasis of tumor cells [138, 139].

One inflammatory mediator relevant in EMT is TGF-β, as demonstrated by its role in embryogenesis, fibrosis, and tumor development in various EMT models [137, 140–142]. SMAD2, SMAD3, and SMAD4 mediate EMT modulation via TGF-β signaling [137], as shown by EMT inhibition in SMAD3-deficient mice and by SMAD2-, SMAD3-, or SMAD4-dominant negative constructs *in vitro* [143, 144]. Extensive evidence supports the notion that EMT can be induced by proinflammatory cytokines. TNF-α and IL-6 may synergistically nudge the TGF-β signaling pathway towards EMT progression (Figures 1(b) and 1(c)) [21, 145]. Both cytokines promote NF-κB activation, which regulates the expression of transcription factors involved in EMT, orchestrating the effects of Snail1, Snail2, Twist, ZEB1, and ZEB2 [146, 147]. Moreover, IL-6 induces cell invasiveness in EMT, through increased vimentin and downregulated E-cadherin expression, both mediated by the JAK/STAT3/Snail signaling pathway, as shown in head and neck cancer [148].

Finally, ROS production can promote EMT [149]; therefore, exposing kidney epithelial cells to ROS induces TGF-β expression, the SMAD signaling pathway, and EMT, whereas antioxidants inhibit these processes [150].

4.4. Inflammation-Associated Angiogenesis. Angiogenesis comprises the processes leading to the generation of new blood vessels from an existing vascular network. Angiogenesis in cancer development is important because the new blood vessel network penetrates and supplies nutrients and oxygen to tumor cells. Several angiogenic factors secreted by tumor cells have been identified, in particular vascular endothelial growth factor (VEGF) that is expressed in response to cytokines and growth factors, as shown in Figures 1(c) and 1(d) [151]. Moreover, characterization of tumor-associated macrophages (TAM) obtained from metastatic lymph nodes (MLN) in an animal model of melanoma has shown that MLN are constituted predominantly by $TIE2^+/CD31^+$ infiltrating macrophages. This subpopulation significantly overexpresses VEGF and is directly related to angiogenesis [152].

Fajardo et al. showed that TNF-α might have a double-edged role in angiogenesis, depending on the dose used. High TNF-α doses inhibited angiogenesis in mice subcutaneously implanted with an angiogenesis disc-system, an experimental strategy used to induce new blood vessels, while low doses promoted vascularization of the area [153]. The antiangiogenic effect of TNF-α is related to downregulation of $\alpha v\beta 3$ and the angiotensin signaling pathway [154], while proangiogenic responses have been associated with increased VEGF, VEGFR, IL-8, and FGF expression [155].

On the other hand, low TNF-α levels increase tumor growth, induce angiogenesis of diverse tumors in mice, and induce a subpopulation of tumor-associated myeloid cells coexpressing endothelial and myeloid markers with proangiogenic/provasculogenic properties [156].

The tumor source of TNF-α can be derived from myeloid or tumor cells and through an autocrine activation can stimulate tumor growth and angiogenesis [157]. Likewise, tumors derived from TNF-α knockdown cells have a well-circumscribed phenotype, with low vascularization and less invasiveness [157].

Another relevant angiogenic factor is IL-6; high levels correlate with VEGF content in colorectal and gastric cancer [158, 159]. Moreover, IL-6 induces VEGF expression in a dose-dependent manner in gastric cancer cell lines [160]. Similarly, IL-6 promotes angiogenesis by activating

the STAT3 pathway in cervical cancer [161]. Together, IL-6 secretion and the subsequent STAT3 phosphorylation are involved in the upregulation of angiogenic mediators, such as VEGF, HIF1α, the VEGFR2 coreceptor, and neuropilin 2 (NRP2) [162, 163]. In xenograft models of ovarian cancer, reduced tumor neovascularization, TAM infiltration, and chemokine production were demonstrated after a challenge with siltuximab, a high-affinity anti-IL-6 antibody [77].

A proangiogenic effect has also been attributed to TGF-β [88]. High TGF-β levels in tumors correlate with angiogenesis in prostate cancer [164]. In addition, TGF-β levels correlate with VEGF expression in gastric carcinoma [165]. These data are consistent with the defective vasculogenesis shown in TGF-β1 knockdown mice [166].

On the other hand, anti-inflammatory IL-10 has been suggested to have an antiangiogenic role in several cancer models [167, 168]. Overexpression of mIL-10 in the KOC-2S tumor cell line had little effect on the VEGF-hyposecretory phenotype, suggesting that mIL-10-mediated inhibition of angiogenesis is mediated by VEGF [169].

4.5. Inflammation-Associated Metastasis. Metastasis is a process characterized by neoplastic cell spread to another organ of different origin. During metastasis, the cells invade blood and lymphatic vessels and circulate through the bloodstream, with subsequent retention in another organ, generating a new tumor focus.

The metastatic cascade is modulated by the action of several cytokines released by surrounding cells such as tumor associated macrophages, infiltrating lymphocytes, and cancer associated fibroblasts, promoting tumor cell evasion and dissemination; this process is depicted in Figure 1(d). The influence of TNF-α has been investigated in various experimental animal models. Administration of this cytokine leads to a significant increase of the number of lung metastases [170, 171]. Kim et al. proposed that tumor cells activate myeloid cells to generate a microenvironment favorable for metastasis. In Lewis lung carcinoma (LLC) cell conditioned-medium, high levels of IL-6 and TNF-α were induced in bone marrow-derived macrophages [172]. TNF-$\alpha^{-/-}$ but not IL-$6^{-/-}$ mice injected with LLC cells showed improved survival and reduced lung tumor multiplicity, suggesting a critical role of TNF-α in LLC metastasis [172]. In accordance with these data, studies show that the use of anti-TNF-α antibodies aids in decreasing metastasis [4, 173]. IL-6, in turn, is upregulated in various tumors and has been implicated in the capacity of cancer cells to metastasize to bone [148, 174, 175].

In contrast, IL-10 displays an antitumoral function. Restitution of IL-10 in the A375P human melanoma cell line, which does not produce endogenous IL-10, using a vector containing murine IL-10 cDNA, reverted tumor growth and lung metastases. This evidence suggests that IL-10 production by tumor cells inhibits metastasis [167].

There is a strong relationship between EMT and metastasis, suggesting that, in the early stages of the metastatic cascade, EMT enables migration and intravasation of tumor cells [176]. For this reason, inflammatory mediators involved in EMT, in particular TGF-β, might play an important role in promoting metastasis [138].

5. Colorectal Cancer and Inflammatory Bowel Disease

Colorectal cancer is the third-most frequent cancer worldwide, with a higher incidence in developed countries [177]. A mortality rate of about 9% has been reported for both men and women, with 5-year survival between 74% and 59% for early stages (stages I to IIC) and 6% for stage IV [178].

Today it is widely accepted that IBD patients have a higher risk of CRC especially ulcerative colitis (UC) and to a much lesser extent Crohn's disease (CD). In a population-based study in the United States, standardized incidence ratios (SIR) of 2.4 (95% IC 0.6–6.0) in extensive UC or pancolitis and 1.9 in CD (95% IC 0.7–4.1) were reported [8]. The prevalence of CRC in UC patients in the Asia-Pacific region ranges from 0.3 to 1.8% [179]. In a Japanese study, poorer survival was observed in patients with ulcerative colitis-associated colorectal cancer as compared to sporadic colorectal cancer patients in advanced stages [180].

Risk factors involved in this process include a greater extent of compromised tissue and sustained disease duration with an onset of more than 7 years, with risk increasing 0.5–1.0% per year [181]. Another risk factor is concomitant primary sclerosing cholangitis (PSC) and UC, with an OR 4.79: 95% CI (3.58, 6.41) [182].

As noted previously, several types of cancer are associated with chronic infections (Table 1). The IBD are multifactorial pathologies involving changes in the microbiota, possibly attributable to pathogens such as *Mycobacterium avium paratuberculosis* and adherent-invasive *Escherichia coli* [183]. These pathogens can induce an inflammatory response [184–186], which may be associated with higher risk of carcinogenesis; however, more studies demonstrating the chronicity of these infections in IBD patients and their potential role in carcinogenesis are needed.

Various murine models of colitis-associated cancer (CAC) [187] have elucidated much of the carcinogenic process, such as a genetic model of IL-10-deficient mice that develop spontaneous colitis and colonic neoplasms [44] and a DSS-induced colitis and carcinoma model. DSS is a mucosal irritant that induces damage similar to that seen in UC patients, and, through a dose-repeated regimen, DSS-exposed mice develop tumors [188, 189]. An additional chemically induced murine model involves an azoxymethane (AOM) stimulus combined with repeated DSS doses. AOM is a mutagenic agent favoring mutation of the β-catenin protooncogene, inducing localization to the nucleus and increasing iNOS and cyclooxygenase (COX-2) expression [190, 191]. Through the animal models, we have learned that inflammatory cytokines, chemokines, and growth factors play crucial roles in CAC development. However, these models have limitations, as they do not always represent the complexity of the mechanisms involved in CRC-IBD patients [187].

In IBD, many inflammatory cytokines are involved in carcinogenesis, such as TNF-α and IL-6 (Table 2). In untreated UC patients, mucosal TNF-α levels correlate with the degree of swelling [192]. Furthermore, high IL-6 levels have been observed in intestinal biopsies from active IBD patients [193],

and murine models have demonstrated a crucial role for these two relevant proinflammatory cytokines in the initiation and progression of CAC [33, 194].

As noted above, proinflammatory cytokines can induce the generation of RONS, a process that has been observed in IBD patients [115], increasing the risk of carcinogenesis [195] by promoting oxidative stress-mediated DNA damage [19]. High ROS levels induced by chronic inflammation have been associated with early p53 mutations in CAC, distinguishing it from sporadic colorectal cancer, in which these mutations have been identified in later stages of malignancy [196]. Thus, the mutagenic potential of RONS, together with early mutations of the p53 tumor suppressor gene, has the potential to increase the cumulative risk associated with genetic alterations predisposing to carcinogenesis in UC patients.

There is abundant evidence for the role of EMT in CAC progression and the participation of TGF-β in EMT [38]. Patients with IBD or CRC show elevated TGF-β levels [197, 198]. In an IL-10-deficient CAC murine model, incidence of colorectal carcinoma was 65% at the age of 10–31 weeks, and plasma TGF-β levels were higher than in their wild-type littermates [44]. Through *in vitro* assays, a well-differentiated colon carcinoma cell line LIM1863 was shown to undergo EMT conversion with a migratory monolayer phenotype in response to TGF-β. Moreover, TNF-α stimulates IL-8 expression, which in turn accelerates TGF-β-induced EMT [21]. Therefore, a proinflammatory stimulus favors the invasive properties of CAC, potentiating EMT.

As previously detailed, angiogenesis is a relevant process in carcinogenesis. Mucosal tissue from IBD patients shows higher microvessel density, a process associated with increased expression of VEGF-induced inflammation [22, 199]. Concomitantly, the CAC mouse model replicated the higher VEGF activity, and blockade of VEGFR2 suppressed tumor development, angiogenesis, and cell proliferation [200].

Furthermore, in an experimental murine cancer metastasis model in which tumor growth was stimulated by bacterial lipopolysaccharide (LPS) injection, TNF-α-induced NF-κB signaling in tumor cells was essential for the generation of metastasis. Moreover, NF-κB blockade resulted in reversion of LPS-induced tumor growth [20]. Taken together, these effects of NF-κB signaling indicate that it is a decisive pathway for driving metastasis.

A recently described molecule involved in metastasis is periostin, an extracellular matrix protein secreted in response to mechanical stress and tissue repair by pericryptal and cancer associated fibroblasts (CAFs). Periostin is expressed in invasive front of colon carcinoma, suggesting its participation in tumor growth [201]. Periostin expression dramatically enhances metastatic growth of colon cancer by both preventing stress-induced apoptosis in cancer cells and augmenting endothelial cell survival to promote angiogenesis [202].

The inflammatory process associated with carcinogenesis in CAC is not limited to the above-mentioned cytokines. Other inflammatory mediators are also involved, such as the proinflammatory cytokine IL-17, which was found to be elevated in the mucosa and serum of active IBD patients [203]. Furthermore, IL-17 is overexpressed in tumors from CAC patients and is associated with angiogenesis and poor prognosis markers [46]. The protumorigenic role of IL-17 has also been observed in a IL-17-deficient mouse model of CAC induced with AOM and DSS, where minor tumor formation and a decrease in proinflammatory markers were found for the IL-17-deficient mice as compared to wild-type mice [204].

Another proinflammatory cytokine with a role in CAC is IL-21, which is elevated in the mucosa of IBD patients and in the CAC mouse model [49]. Furthermore, blockade of the IL-21 signaling pathway reduces tumor development and mucosal microenvironment inflammation [49].

Interferon-γ (IFN-γ) is a proinflammatory cytokine with pleiotropic functions [205]. Increased numbers of IFN-γ positive cells have been observed in IBD patients, especially Crohn's disease [27], possibly contributing to a chronic inflammatory setting. Moreover, IFN-γ-deficient mice did not develop DSS-induced colitis [28]. In early IBD pathogenesis, IFN-γ plays an important role in increasing paracellular permeability in T84 epithelial cells by inducing endocytosis of tight-junction (TJ) proteins occludin, JAM-A, and claudin-1 [29]. In an IL-10-deficient model, enterocolitis and tumor formation were dependent on the participation of IFN-γ, as blockage with a neutralizing antibody prevented colitis and cancer in young mice (less than 3 weeks old). However, this effect was not seen in mice older than 3 months, emphasizing the role of IFN-γ as an early inducer of inflammation [95].

In an AOM/TNBS-CAC murine model, Osawa et al. showed that IFN-$\gamma^{-/-}$ mice developed higher numbers of tumors than wild-type or IL-4$^{-/-}$ mice. This points to the antitumor immune response of IFN-γ [30]. In patients with UC-associated cancer and a group of UC patients with chronic severe inflammation, the IFN-inducible gene family 1-8U was overexpressed. However, the consequences of increased IFN-γ expression in UC and its contribution to carcinogenesis remain unclear [31].

Other molecules induced by IFN-γ have been also observed in IBD patients, such as IL-18 and IL-18 binding protein (IL-18BP), which have been furthermore associated with inflammation and cancer [32].

Interleukin 8 (IL-8), a member of the neutrophil-specific CXC subfamily of chemokines with the ELR (Glu-Leu-Arg) motif, acts as a chemoattractant to neutrophils during acute inflammatory response [206]. Increased levels of this chemokine have been reported in IBD patients [207], correlating histologically with areas of active inflammation [208], mainly associated with neutrophils and macrophages [209]. Additionally, colon cancer cells also express IL-8 [210]; in sporadic cancer, higher levels of this cytokine were observed in tissue from moderately and poorly differentiated as compared to well-differentiated tumors [211]. In addition, IL-8 levels are directly correlated with metastatic potential in colon cancer cell lines [210]. Overexpression of IL-8 in HCT116 and Caco2 cell lines results in increased proliferation, cell migration, and invasion, while in a tumor xenograft model, IL-8-overexpressing cells formed larger tumors and showed higher microvessel density [41]. This *in vivo* effect of IL-8 on angiogenesis is supported by a study using primary cultures of human intestinal microvascular endothelial cells,

Table 2: Significance and role of cytokines in tumorigenesis.

Cytokines	Colitis-associated cancer (references)	CCA (references)
TNF-α	Tumor-promoting role in various stages of carcinogenesis. Related to RONS generation in IBD patients, promoting oxidative stress-mediated DNA damage. Stimulates TGF-β-induced EMT. Induces secretion of VEGF by human fibroblasts, promoting angiogenesis. Induces NF-κB signaling, a decisive pathway in driving metastasis in a model of CAC [19–22].	Essential for bile duct epithelial cell proliferation. Impairs epithelial barrier function. Disrupts cholangiocyte tight-junction and influences the aggravation of bile duct cholestasis. Induces a DNA/RNA-editing enzyme (AID) in CCA cells, resulting in somatic mutation of several tumor-related genes and leading to cholangiogenesis. EMT induction in CCA cells *in vitro* [23–26].
IFN-γ	Increases in IFN-γ^+ cells have been observed in IBD patients. Deficient mice did not develop DSS-induced colitis. Increases paracellular permeability in early IBD pathogenesis. Deficient mice developed higher numbers of tumors, suggesting an antitumor immune response of IFN-γ. In patients with UC-associated cancer and a group of UC patients with chronic severe inflammation, the IFN-inducible gene family 1-8U was overexpressed. Induces IL-18 and IL-18 binding protein (IL-18BP) in IBD, which have been also associated with inflammation and cancer [27–32].	Reduces transepithelial electrical resistance. Alters cholangiocyte tight-junction, leading to aggravation of bile duct cholestasis [24].
IL-6	Induces oxidative stress. A critical tumor promoter during early CAC tumorigenesis. TAM-derived IL-6 contributes to CAC in animal models. CRC patients present with high levels of IL-6 and VEGF [19, 33–35].	Cholangiocyte and CCA cells can be activated by proinflammatory cytokines through the NF-κB-dependent pathway, leading to overproduction of bile duct epithelium growth factor, thus promoting cancer initiation and progression [36, 37].
TGF-β	Induces CAC progression, promoting EMT. In later stages of carcinogenesis, it promotes tumor growth by creating an immunotolerant tumor environment [38, 39].	Promotes proliferation of bile duct epithelial cells and induces EMT-mediated tumor aggressiveness [23, 40].
IL-8	Colon cancer cell lines overexpressing IL-8 show enhanced proliferation, migration, and angiogenesis. IL-8 induced by TNF-α accelerates EMT [21, 41].	Secreted by cholangiocytes in response to proinflammatory cytokines and together with MCP-1 and CCL-28 promotes leukocyte adhesion and retention in injured biliary epithelial cells. Injured cholangiocytes then release IGF-1 and VEGF, which can stimulate CCA cell growth [42, 43].
IL-10	IL-10$^{-/-}$ mice develop colitis and colorectal cancer, similar to IBD-associated cancer in humans [44].	CCA can activate macrophage polarization into M2 phenotype through the STAT-3 pathway, leading to IL-10, VEGF-A, TGF-β, and MMP-2 production [45].
IL-17	Overexpressed in tumors from CAC patients and is associated with angiogenesis and poor prognosis markers. Secreted in tumors by macrophages/monocytes CD68+; Th17 and Treg FOXP3$^+$IL17$^+$ cells [46, 47].	Tumor-infiltrating lymphocytes IL-17$^+$ are found in CCA intratumoral areas and correlate with lymph node metastasis, intrahepatic metastasis, and advanced stages [48].

Table 2: Continued.

Cytokines	Colitis-associated cancer (references)	CCA (references)
IL-21	Enhanced in mucosa of IBD patients and in the CAC mouse model. Blockade of IL-21 signaling reduces tumor development and mucosal microenvironment inflammation [49].	No available references for this cytokine in CCA.

which respond to IL-8 through the CXCR2 receptor, eliciting an angiogenic response [212].

These findings illustrate the complex role of cytokines in the various events associated with the development of CAC. Therefore, controlling the inflammatory process early in IBD is important for reducing risk of colorectal cancer.

6. Primary Sclerosing Cholangitis- (PSC-) and Liver Fluke-Associated Cholangiocarcinoma (CCA)

CCA is a malignant neoplasm originating from the epithelial cells lining the intra- or extrahepatic biliary ducts. It is the second-most frequent liver cancer worldwide, after hepatocellular carcinoma. Five-year survival is about 10%. In the United States, incidence of CCA in the Hispanic population is 2.8 per 100,000; in Asians, 3.3 per 100,000; and in non-Hispanic Caucasians and African-Americans, 2.1 per 100,000 [213]. However, incidence varies widely, from the highest reported rate of 113 per 100,000 in the Khon Kaen province of Thailand to as low as 0.1 per 100,000 in Australia [214, 215].

There are several factors that increase the risk for CCA, including primary sclerosing cholangitis, parasitic infection, biliary-duct cysts, hepatolithiasis, viral infection, and toxins [23, 216]. Primary sclerosing cholangitis (PSC) is characterized by inflammation and fibrosis of biliary ducts leading to biliary tract stricture. The cumulative lifetime incidence of CCA in PSC is around 20% [217]. More than 50% of patients with PSC develop CCA simultaneously or within 1 year of diagnosis [218]. The incidence of CCA after PSC diagnosis has been reported in several studies at around 0.5–1.5% per year [217–219]. CCA must be suspected in any new PSC patient presenting with jaundice, suggesting chronic inflammation of the bile duct.

Opisthorchis viverrini (*O. viverrini*) and *Clonorchis sinensis* (*C. sinensis*) have been classified by the International Agency for Research on Cancer (IARC) as Group I (carcinogenic in humans) [220] and as the most common risk factors for CCA, especially in East and Southeast Asia [221, 222]. The high incidence of *O. viverrini* infection, which is due to the custom of eating raw fish containing the infectious stage of the parasites, was found to be correlated with the high prevalence of CCA in the northeastern part of Thailand [221]. PSC, hepatolithiasis, and choledochal cysts are the risk factors for CCA in areas where liver fluke is not endemic in Thailand [215]. In addition, biliary ascariasis caused by *Ascaris lumbricoides* infection in China, India, and some areas of South America has also been reported in association with CCA development [223, 224].

Infection with hepatitis viruses can generate hepatocellular carcinomas, especially hepatitis B, in which more than 80% of cases develop cancer [225]. It is becoming more accepted that both hepatitis B and hepatitis C viruses may be associated with biliary inflammation and can cause CCA. Approximately 13.8% and 1.9% of CCA patients have positive findings for hepatitis B and hepatitis C, respectively [226].

Other etiologies that may or may not cause bile duct obstruction but result in the chronic inflammation of biliary epithelial cells are proposed CCA risk factors, including gallstone formation, choledochoenteric anastomosis, and chemical and radiation exposure [23].

CCA, like many other cancers in that its carcinogenesis is a multistep process, requires interaction between mutated biliary epithelial cells and environmental factors. Many hallmarks of cancer have been proposed, and the list has been continually updated over the years [7]. The genes involved in controlling these properties have been found to be mutated in cancer patients. In CCA, several protooncogenes including K-ras [227–229], c-erbB-2, and c-Met [230]; tumor suppressor genes, that is, p53; and antiapoptotic genes such as Bcl-2, Bcl-X(L), and Mcl-1 [231] are mutated. In PSC-mediated CCA, the mutation was detected in the promoter, leading to the overexpression of p16INK4a and p14ARF cell cycle regulators [232].

During the genesis of CCA, both PSC and parasitic infections cause cholestasis and chronic inflammation of the bile duct, which can induce the epithelial cells to produce a variety of cytokines including IL-6, IL-8, TGF-β, TNF-α, platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) (Table 2) [23]. The release of IL-6, TGF-β, TNF-α, and PDGFA is essential for bile duct epithelial cell proliferation. The production of PDGFA and the overexpression of its receptors during cholangiocarcinogenesis in *O. viverrini*-infected hamsters indicate the potential of these molecules to downregulate many antiproliferative factors and promote the angiogenesis pathway [233]. In addition, PDGFA expression in CCA tissue and serum is correlated with patient survival time and has been proposed as a marker of poor prognosis [234].

TNF-α and IFN-γ, which are cytokines released during chronic inflammation, can cause alteration of biliary barrier function [24], whereas proinflammatory cytokines alter cholangiocyte choleretic activity [42, 43]. When cholangiocytes are exposed to these cytokines, they respond by secreting other molecules such as IL-8, MCP-1, and CCL-28 that can promote leukocyte adhesion and retention at the site of inflammation, leading to more damage of biliary cells. The injured cholangiocytes can release insulin-like growth

factor-1 (IGF-1) and VEGF to stimulate CCA cell growth and angiogenesis, respectively [235–238].

TNF-α can activate increased expression of AID (activation-induced cytidine deaminase, a member of the DNA/RNA-editing enzyme family) in CCA-derived cells, but not in PSC-derived epithelial cells [25]. AID results in the generation of somatic mutations of many tumor-related genes, including p53, c-Myc, and CDKN2A (or INK4A/p16) promoter sequences. This finding suggests a connection between chronic inflammation and tumorigenesis via the mutagenic activity of AID [25]. In addition, NF-κB activation in cells by chronic inflammation-derived cytokines might lead to the activation of active transcription factors translocating into the nucleus and regulating the expression of IL-6, TNF-α, and several growth factors which can change the microenvironment for tumor promotion [36]. Moreover, the release of nitric oxide with the formation of 3-nitrotyrosine and other reactive oxidants can inhibit the DNA-repair process, which allows for oxidative DNA damage to cells and thus promotes tumor formation [239].

Cholangiocytes and CCA cells do not act alone but are surrounded by several types of cells, generally known as microenvironmental cells. Fibroblasts are the main microenvironmental cells, and their function in stimulating the acquired hallmark capabilities of cancer cells is well-known [240]. Activated CCA-associated fibroblast phenotypes were found to show increased expression of α-SMA [241]. Interestingly, these fibroblasts were isolated from CCA tissues obtained from patients and mapped for the specific gene expression pattern resulting in the expression of several cancer-promoting proteins [242]. Researchers have since identified several substances that can be produced by CCA-associated fibroblasts, including periostin, hepatocyte growth factor (HGF), tenascin-C, and CXCL-12 [243, 244]. Interestingly, these soluble factors are involved in several tumorigenic properties leading to the progression and metastasis of the cancer. These findings suggest that fibroblasts, their secreting products, and the activated pathways in the cancer cells could be promising targets for attenuation of disease progression [243, 245].

Many immune cells are known to surround cancer cells, with detrimental or beneficial effects on cancer progression, depending on the profile of substances secreted into the tumor microenvironment. The substances secreted from CCA cells were studied *in vitro* with human macrophages, and the results exhibited M2 polarization of macrophages as well as overproduction of cytokines and other bioactive molecules, including IL-10, VEGF-A, TGF-β, and matrix metalloproteinase- (MMP-) 2 [45]. In intrahepatic CCA, the tumor-infiltrating lymphocytes IL-17$^+$ and FOXP3$^+$, CD66b$^+$ neutrophils, and microvessels were predominantly found in the intratumor area, whereas CD8$^+$ lymphocytes were most abundant in the tumor invasive front [48]. Although IL-17 levels have never been reported for CCA, this study suggested for the first time that intratumor IL-17$^+$ lymphocytes and neutrophils could be used as a marker of poor prognosis in CCA.

TGF-β was studied with CCA cell lines, and the results demonstrated the potential of TGF-β to induce EMT-mediated cancer progression via the Snail transcription factor, leading to increasing levels of vimentin, S100A4, collagen type 1, and MMP-2 production [40]. EMT level is closely associated with aggressiveness of the disease and could be proposed as a marker of poor prognosis. Moreover, TNF-α has been recently reported to have the ability to induce EMT of CCA cells [26].

In conclusion, the chronic inflammation-driven cytokines released from biliary cells, fibroblasts, or immune cells into the microenvironment of the bile duct epithelium may facilitate cell immortalization, evasion of apoptosis, and autonomous proliferation in untransformed cells, leading to the development of CCA [23]. In addition, cytokines may help activate invasion, metastasis, and EMT-mediated CCA progression.

7. Conclusion

The tumor microenvironment formed by stromal cells, infiltrating immune cells, and tumor cells contains factors that can promote carcinogenesis. Ample evidence supports the involvement of cytokines in events leading to the initiation, promotion, invasion, and metastasis of cancer (Figure 1). In a chronic inflammatory process, cytokines such as TNF-α and IL-6 induce the generation of free radicals that can damage DNA, potentially causing mutations that lead to tumor initiation. Tumor growth is also favored by proinflammatory cytokines that stimulate cell proliferation and reduce apoptosis, while anti-inflammatory cytokines, such as IL-10 and TGF-β, contribute to tumor immune evasion. The invasive properties of tumors are related to the activation of the epithelial-mesenchymal transition program triggered by TGF-β and enhanced by proinflammatory cytokines, such as TNF-α and IL-6. Proinflammatory cytokines also play an important role in angiogenesis and metastasis. In the latter, chemokines such as IL-8 have an important role in cell migration to other tissues.

Although we observed that many cytokines contribute to carcinogenesis, their pro- or antitumoral roles depend on the balance of these different inflammatory mediators and the stage of tumor development. For this reason, studying the role of these mediators in different tumors or stages of development is essential for designing new personalized treatments using these potential therapeutic targets.

In this line, the potential role of cytokines has been reported, as a diagnostic marker for cancer. The determination of the serum levels of cytokines, such as IL-6 or IL-10, might be associated with a tumorigenic process or poor prognosis [69, 105]. However, further prospective studies are needed to determine trusted cut-off values of circulating cytokine to establish a direct relationship with cancer.

In the field of therapy, several clinical trials have been implemented in order to evaluate inhibitors of cytokines receptors or neutralizing antibodies that prevent the sustained exposure to these inflammatory mediators that promote tumor progression [80, 103]. On the other hand, from the findings of Coley [56], who associates an infectious process with the control of tumor progression, arises

the idea to cause an acute inflammation to activate antitumor response mechanisms [58].

While progress has been made in the understanding of the mechanisms of these cytokines in the tumorigenic process, establishing a relationship between cytokines expression and disease progression, survival, and response to therapy remains a major challenge.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Glauben Landskron and Marjorie De la Fuente are contributed equally to this paper.

Acknowledgments

The figures were produced using Servier Medical Art from www.servier.com. Funding was received from FONDECYT 1120577 (MAH) and CONICYT REDES130037 (MAH).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 282495, 6 pages
http://dx.doi.org/10.1155/2014/282495

Review Article

Involvement of the Circadian Rhythm and Inflammatory Cytokines in the Pathogenesis of Rheumatoid Arthritis

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Received 5 March 2014; Accepted 24 April 2014; Published 8 May 2014

Academic Editor: Mizuko Mamura

Among the symptoms of patients with rheumatoid arthritis (RA), joint stiffness is influenced by diurnal rhythm and reaches peak in the morning, which is a common complaint and reflects the circadian nature of disease manifestation. In addition, inflammatory cytokines, which reach peak secretion early in the morning are major players causing the morning stiffness. In this review, we explore the link between the circadian clock and inflammation, focusing on the interactions of various clock genes with the immune-pathways underlying the pathology of rheumatoid arthritis.

1. The Biological Origins and Regulation of the Body Clock

The word "circadian" is defined as the period of physiological function and behavior of the organism, which is about 24 hours in an environment with no external constraints. The circadian rhythm has been shown to be involved in a number of physiological processes, including sleep/awakening, body temperature regulation, hormone secretion, division and proliferation of cells, and gastrointestinal function. Although we normally think of the circadian rhythm as 24 hours, it is in fact slightly longer in humankind [1, 2]. As a result, organisms need to correct their biological clock daily using external cues, the most effective of which is light stimulus. While other organisms use the same organ for both light reception and setting of the biological clock [3, 4], mammals have evolved separate locations for these two functions. The controlling center for rhythm oscillation in mammals is the hypothalamic suprachiasmatic nucleus (SCN), located at the base of the brain in front of the pituitary gland. The SCN is too deep in the brain to sense light cues directly and instead receives external cues via the optic nerve that transmits signals from the retina and combines the function of both light-dark tuning and rhythm transmission to act as a master clock of the whole body [5].

The role of the SCN in coordinating body rhythm was first shown in rodents, where removal of the SCN was observed to cause a collapse in the rhythmicity of behavior and endocrine activity, suggesting that this structure may be the underlying circadian pacemaker for physiological and behavioral activities in mammals [6]. However, subsequent studies found that the liver and lung cells maintain their own rhythm when cultured outside the body and without input from the SCN. Thus, it has become apparent that tissues and cells can provide their own peripheral rhythm, similar to that provided by the brain, which is maintained by the activities of the clock genes [6, 7].

The clock genes manage rhythm and time in a dual and hierarchical manner. The rhythm signals propagated from SCN are subject to a feedback loop provided by core clock genes including *CLOCK* (circadian locomotor output cycles kaput), *BMAL*1 (brain and muscle ARNT-like-1), *PER* (period) and *CRY* (cryptochrome), and orphan nuclear hormone receptors *REV-ERB* alpha (encoded by *Nr1d1*) and *Ror alpha*. The circadian expression of these genes is regulated through E/E′ boxes (CACGTG/CACGTT

sequence), REV-ERBα/ROR response element (RRE), and DBP/E4BP4 binding element (D box) in their promoter regions. First, the CLOCK and BMAL1 proteins form a complex that binds to the E/E′ boxes present in the promoter regions of the *Per*1–3, *Cry*1-2, *Nr1d*1, and *Ror alpha* genes, thus regulating their transcription. The PER and CRY proteins, in turn, form a complex in the cytoplasm and relocate to the nucleus where they inhibit the genes induced by the CLOCK/BMAL1 protein complex, including their own transcription [6]. Second, REV-ERB and ROR compete on RRE element on the promoter region of *BMAL*1 to regulate its transcription [8]. In addition, DBP (D site of albumin promoter binding protein) promotes and E4BP4 (E4-binding protein 4) suppresses the transcription of *PER* by binding to the D box present in the promoter region [9, 10]. Although not all clock genes directly respond to CLOCK/BMAL1, this core control mechanism is repeated in about a 24-hour period. Importantly, circadian transcriptional circuits are governed by two design principles [11]: regulation of E/E′ boxes and RRE element follows a repressor-precedes-activator pattern, resulting in delayed transcriptional activity, whereas regulation of D box follows a repression-antiphasic-to-activator mechanism, which generated high-amplitude transcriptional activity. In addition, recent study has shown that circadian modulation of RNA polymerase II recruitment and chromatin remodeling occurs on a genome-wide scale [12], so that there are various mechanisms for generating rhythmicity of gene expression.

2. Melatonin and Inflammatory Cytokines Tune the Circadian Regulation

In the daytime, light-initiated signals are transmitted via the SCN to the pineal gland through the upper cervical ganglion. Melatonin, a hormone that mediates circadian rhythm adjustment, is produced by the pineal gland at night. Light stimulus causes an increase in the secretion of cortisol, serotonin, and dopamine, while suppressing melatonin, norepinephrine, and acetylcholine. Serum melatonin levels are normally undetectable in the daytime but are significantly higher during the night, in the absence of optical stimulation [13–16].

How might melatonin be involved in the pathogenesis of rheumatoid arthritis? As compared with healthy subjects, melatonin secretion at midnight is significantly increased in RA patients [17], and melatonin serum levels in the morning are higher in RA patients with shorter disease duration [18]. Inflammatory cytokines including IFN-γ (interferon-gamma), IL (interleukin)-1, and IL-6 are all secreted from human peripheral blood mononuclear cells in response to melatonin stimulation, and in fact, melatonin is detected in RA synovium tissue macrophages and joint fluid [19]. These studies seem to suggest that melatonin has an adverse effect on arthritis; on the other hand, melatonin inhibits the activity of MMP (matrix metalloproteinase)-9, which is involved in joint destruction in RA patients [20]. Thus, further study is needed to determine the effects of melatonin on joint destruction.

Studies in recent years have helped elucidate the influence of inflammatory cytokines on circadian mechanisms. For example, activation of the immune system counteracts infection and increases resistance to pathogens by inducing slow wave sleep, presumably via the production of inflammatory cytokines such as TNF-α, IL-2, or IFN-γ that are known to induce such sleep [21]. Indeed, LPS (lipopolysaccharide), which is the major component of the outer membrane of Gram-negative bacteria, stimulus also uniformly increases the secretion of these cytokines. Conversely, immune cells exhibit enhanced proinflammatory responses and LPS-induced IL-6 release if the circadian rhythm is disrupted by an external change in the light-dark cycle [22]. Interestingly, LPS-dependent secretion of TNF-α is significantly higher at night compared to day and is further enhanced by melatonin stimulation. Through their ability to promote sleep, cytokines are involved in the generation of an inner rhythm that controls the secretion of growth hormone, prolactin, and cortisol [23].

How is the relation between sleep and rheumatoid arthritis that is well known to be a chronic inflammatory disease? RA patients often exhibit sleep disorders classified as a nocturnal awakening type, and this type of disorder is characterized by a significant reduction in sleep efficiency and a significant increase in waking periods after sleep onset. Questionnaire studies of patients with sleep disorders report a "decline in the quality of sleep in patients with RA" as quantified by the Pittsburgh Sleep Quality Index [24, 25], and "excessive somnolence trend during the day in patients with RA" by the Epworth sleepiness scale [26]. Further, increased RA disease activity has been reported to correlate with sleep disorders, and the correlation is somewhat stronger in women and is mitigated by age [27, 28]. In addition, shift work was associated with risk of RA in women [29]. This could be supported by the concept that the body clock not only impacts on arthritic symptoms but is also involved in the pathogenesis of RA [16].

In RA, a major source of proinflammatory cytokines is immune cells such as T cells and macrophages. Spleen, peripheral lymph nodes, and peritoneal fluid-derived macrophages operate autonomous circadian clockworks even ex vivo, and spleen cells secrete TNF-α and IL-6 in a circadian manner under the stimulation with bacterial endotoxin [16, 30]. Further, endotoxin-induced cytokine and chemokine production was significantly affected by BMAL1 and REV-ERBα expression. Since the nuclear receptors RORα and REV-ERBα are key molecules that modulate *BMAL*1 transcription in the process of feedback-regulation of circadian genes, *REV-ERB*α knockout condition represented the loss of circadian gating of endotoxin response, especially in the release of IL-6, through macrophages [31], and agonists or overexpression of REV-ERBα inhibited the expression of Ccl2, also named monocyte chemotactic protein-1 (MCP-1), by binding to their promoter regions in murine macrophage cell line [32]. In addition, REV-ERBs regulate their target genes by inhibiting the functions of distal enhancers that are

selected by macrophage-lineage-determining factors, thereby establishing a macrophage-specific program of repression [33]. This relation between BMAL1 and inflammation was also demonstrated from different angles that *BMAL*1-deleted myeloid cells disrupted differentiation and circadian oscillation of inflammatory monocyte [34].

CD4 positive T cells possess a circadian oscillator that drives rhythmic responses to the stimuli, as manifested by altered cell proliferation and cytokine secretion [16]. Interestingly, it has been shown that T cell numbers and its reactivity were stable during daytime, whereas a significant increase was observed in the late evening and early morning hours ex vivo [35]. These results may explain the portion of etiology for "morning stiffness of joints," a common complaint and one of the best indicators of the condition of RA patients, correlates with the secretion of TNF-α, IL-6, and IFN-γ, whose levels peak from midnight to early in the morning [36], and chronobiology-based approach has been tested and shown to improve the morning symptoms of RA [37, 38].

3. A Pathological Link between RA and the Clock Genes: Synovitis and Wee-1 Kinase

RA is a chronic polyarthritis condition that goes through repeated relapse and remission as the disease progresses. These cycles of elevated inflammation cause deformation and destruction of the joint and irreversible dysfunctions. All joints are affected by RA, large, medium and small. Once activated by inflammation, the mesenchymal cells lining the joint space (synovial cells) begin to proliferate, and inflammatory granulation tissue, called pannus, invades the bone and cartilage, leading to joint destruction. Growth factors, angiogenic factors, and inflammatory lymphocytes work together to promote pannus formation, and joint destruction follows the secretion of MMPs and migration of synovial cells [39, 40]. When we examine cytokine activities, we find that IL-1β and MMPs are deeply involved in osteoporosis of the joint and in the narrowing of the surrounding joint space seen in the early stages of disease onset. In the later stages, TNF-α, IL-1β, and IL-6 are produced and accumulate in the joint cavity as synovitis worsens. Finally, at the peak of arthritis, modification of the inflammatory response by IL-17 is observed [41].

However, lymphocytes are not present in the local pannus during the early stages of RA. In these early stages, joint destruction is promoted by synovial cells under the influence of various cell cycle regulators and transcription factors. For example, synovial cell proliferation is enhanced through the suppression of p21 and overexpression of the transcription factor c-fos [42], while mitotic activity is inhibited through wee-1 kinase [43]. This is a characteristic feature of synovial cells, representing "tumor cell-like proliferation." Indeed when *c-fos* transgenic mice are used in an arthritis model, joint destruction proceeds not as a result of lymphocyte invasion, but by synovial cell proliferation [44]. And, as reported earlier, the expression wee-1 kinase, a G2 /M cell cycle control factor, is increased in mice lacking an essential mammalian timekeeping gene *CRY* [45].

4. Clock Genes and Arthritis; Cry, Per and TNF-α Exacerbate Inflammation

To verify the significance of increased wee-1 expression in *CRY* knockout mice (*CRY*1$^{-/-}$ *CRY*2$^{-/-}$ mice), we utilized a mouse model of arthritis [46]. First, we confirmed the influence of arthritis on clock gene expression using wild type (WT) mice that had been administered anticollagen antibody and LPS and PER2 protein levels in the synovium were monitored. PER2 is usually expressed at night, but in the arthritis model PER2 was highly expressed in the morning. In addition, the phase of *PER*1/2 mRNA expression in spleen lymphocytes was shifted back ~6 hrs, and *BMAL*1 and *Per*1/2 mRNA expression levels were reduced. These observations indicate that the onset of arthritis indeed affects the expression of clock genes *in vivo*.

Next, we examined spleen-derived lymphocytes from *CRY* knockout mice. Peripheral T lymphocytes were found to be constitutively activated, and stimulation of splenocytes by anti-CD3/CD28 antibodies produced higher amounts of TNF-α. In addition, wee-1 protein was overexpressed in the spleens of *CRY* knockout mice. Together, these results suggest that *CRY* knockout mice are ready or primed for arthritis onset. Accordingly, arthritis of the limbs was strongly induced by type II collagen cocktail in *CRY* knockout mice, and this arthritis was suppressed by anti-TNF-α antibodies. Finally, mutual regulation between the TNF-α and CRY genes was demonstrated using luciferase reporter assays.

In addition, recent studies have shown that the absence of *CRY* leads to constitutive activation of protein kinase A, which results in phosphorylation of p65, and thereby ultimately induces NF-κB activation and expression of IL-6 as well as TNF-α [47]. Consistently, expression of *CRY*1 was markedly decreased by administration of melatonin, subsequently aggravated in mouse antitype II collagen antibody-induced arthritis [48]. Further, a phase and amplitude of the clock genes were different between osteoarthritis fibroblast-like synovial cells (OA-FLS) and RA-FLS cells [49]. Thus, these results suggest that clock genes, such as CRY, seem to be deeply involved in both inflammation and arthritis.

Then, can the inflammatory pathways directly affect the expression of clock genes? Table 1 shows the effect of TNF-α on expression of clock genes in mouse and human cells. Using NIH3T3 mouse fibroblast cells, Cavadini et al. reported TNF-α inhibited the expression of *PER*1/2/3, *DBP*, *TEF,* and *HLF*, slightly enhanced *CLOCK* and *REV-ERB*α, and did not affect *BMAL*1, by interfering with E box-mediated transcription of clock genes. And also IL-1β, but not IL-6, seemed to have a biological effect as well as TNF-α [50]. Further, the early effect of TNF-α on expression of the *PER*1 gene is dependent on p38, mitogen-activated protein kinase (MAPK), and/or calcium signaling, whereas its effect on the *DBP* gene is independent of MAPK but dependent on calcium signaling [51]. In human monocytic THP-1 cells, TNF-α enhanced the expression of *BMAL*1 and *CRY*2, whereas it decreased those of *PER*2, *CRY*1, and *REV-ERB*α [52]. Unlike mouse cells, TNF-α did not affect or slightly enhanced the expression of *PER*1 in OA-FLS, RA-FLS, and THP-1 cells [49, 52, 53]. However, TNF-α

Table 1: Effect of TNF-α on the expression of clock genes in mouse NIH3T3, human OA-FLS, RA-FLS, and THP-1 cell line*.

Clock gene	Mouse	Human		
		Fibroblast		Monocyte
	NIH3T3	OA-FLS	RA-FLS	THP-1
Bmal1	Not affected	—	Up	Up
Clock	Slightly up	—	Slightly up	Not affected
Per1	Down (early~3 h: up)	Not affected	Slightly up	Slightly up
Per2	Down	—	Down	Down
Per3	Down	Down	—	—
Cry1	Up	—	Up	Down
Cry2	Slightly down	—	Not affected	Up
Dbp	Down	Down	Down	—
Tef	Down	—	Down	—
Hlf	Down	—	Down	—
E4BP4	—	—	Up	—
Rev-erbα	Slightly up	—	—	Down

*Based on results of the refer 49–53. — denotes no data. OA-FLS: osteoarthritis fibroblast-like synovial cells. RA-FLS: rheumatoid arthritis fibroblast-like synovial cells.

has an inhibitory effect on the expression of DBP and *PER*3 in OA-FLS cells [49]. And we observed, using RA-FLS cells, TNF-α also inhibited the expression of *PER*2 and slightly enhanced CLOCK as well, whereas it significantly increased the expression of *BMAL*1 and *Cry*1 [53]. Interestingly, in TNF-α-stimulated RA-FLS cells, expression of *DBP*, *TEF* and *HLF* was reduced while *E4BP4* was increased, a positive and negative transcriptional regulator of *PER*2, respectively, suggesting another transcriptional regulation of *PER*2 by TNF-α via D box, but not E box [53]. Thus, the proinflammatory cytokines such as TNF-α could affect the expression of clock genes through their inflammatory cascades. In addition, if we recall that *PER*2 knockout mice exhibit increased resistance to apoptosis in thymocytes [54], decreased expression of *PER*2 by TNF-α can also contribute to the resistance of synovial cells to apoptosis and may contribute to the tumor-like growth of the synovium.

Recently, series of observations has made increasingly clear the involvement of clock genes in the pathogenesis of arthritis. Kouri et al. confirmed that BMAL1 protein is markedly localized in the cytoplasm of RA synovium [49]. Interestingly, in mouse model of osteoarthritis (OA), circadian oscillations of *PER*2 transcription are reported to be significantly reduced in cartilage from aged mice [55], and the robust oscillations of *PER*2 transcription are confirmed in cartilage from juvenile mice [56]. These results suggest not only the similarity to human OA that affected individuals increases with age, but also the relation between circadian rhythms and physiology of cartilage formation, that is, also important for joint destruction mechanism in RA.

5. Conclusion

The past 20 years have seen dramatic progress in elucidating the genetic regulation of the body's circadian rhythm. An intricate system of intermolecular interaction modulates the circadian rhythms in cells to an approximate 24-hour cycle, and this cycle is normally maintained and readjusted by both central and peripheral mechanisms. Maintenance of this cycle is important in maintaining a number of homeostatic functions in the body, and dysfunction of this body clock can promote a number of human diseases and pathologies. Since clock genes are highly conserved, it is not surprising that they are responsible for modulating a large number of biological functions. We expect that future research will help us understand not only the various functions of clock genes, but also the strategy to save the primary roles and functions of circadian clock for the human health which is disturbed by a number of factors in the modern society.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 474217, 5 pages
http://dx.doi.org/10.1155/2014/474217

Research Article

Disruption of the Suprachiasmatic Nucleus Blunts a Time of Day-Dependent Variation in Systemic Anaphylactic Reaction in Mice

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Received 6 February 2014; Accepted 7 April 2014; Published 17 April 2014

Academic Editor: Mizuko Mamura

Anaphylaxis is a severe systemic allergic reaction which is rapid in onset and potentially fatal, caused by excessive release of mediators including histamine and cytokines/chemokines from mast cells and basophils upon allergen/IgE stimulation. Increased prevalence of anaphylaxis in industrialized countries requires urgent needs for better understanding of anaphylaxis. However, the pathophysiology of the disease is not fully understood. Here we report that the circadian clock may be an important regulator of anaphylaxis. In mammals, the central clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus synchronizes and entrains peripheral circadian clock present in virtually all cell types via neural and endocrine pathways, thereby driving the daily rhythms in behavior and physiology. We found that mechanical disruption of the SCN resulted in the absence of a time of day-dependent variation in passive systemic anaphylactic (PSA) reaction in mice, associated with loss of daily variations in serum histamine, MCP-1 (CCL2), and IL-6 levels. These results suggest that the central SCN clock controls the time of day-dependent variation in IgE-mediated systemic anaphylactic reaction, which may provide a novel insight into the pathophysiology of anaphylaxis.

1. Introduction

Anaphylaxis is a rapid, potentially fatal, multiorgan system, allergic reaction caused by the excessive release of mediators including histamine and cytokines/chemokines from mast cells and basophils upon allergen/IgE stimulation [1]. It typically causes a number of symptoms including an itchy rash, throat swelling, and low blood pressure associated with low body temperature. A recent nationwide cross-sectional telephone survey in USA estimated that the prevalence of anaphylaxis in the general adult population is at least 1.6% and medications were the most common trigger (35%), followed by foods (32%) and insect stings (19%) [2]. It is also recently reported that an estimated 1 in 300 of the European population at some time in their lives is affected [3]. Given the increased prevalence of anaphylaxis in industrialized countries, more basic research is urgently needed for better understanding of anaphylaxis that leads to efficient prevention of the disease. However, the pathophysiology of anaphylaxis is not fully understood.

In mammals, the internal time keeping system "circadian clock" drives the daily rhythms in behavior and physiology (e.g., sleep-wake cycles, body temperature, blood pressure, and hormonal secretions) that enable the organisms to keep track of the time of day according to daily changes in light intensity [4, 5]. Mammalian circadian clock system consists of the central oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus and peripheral oscillators present in virtually all cell types [4, 5]. Light activates a specific group of photoreceptors in the retina connected to the central SCN

clock which synchronizes and entrains peripheral circadian clock via neural and endocrine pathways. The molecular mechanisms of rhythm generation are cell autonomous, highly conserved in the SCN and peripheral cells, and created and maintained by interlocked transcriptional-translational feedback loops consisting of several "clock genes" and their protein products [4, 5].

We have recently shown that a passive systemic anaphylactic (PSA) reaction in mice exhibits a time of day-dependent variation, relying on the normal activity of a key clock gene, *Period2* (*Per2*) [6], suggesting that the canonical clock gene is required for the daily rhythm generation observed in PSA reaction. However, it remains obscure whether the daily rhythms are generated precisely by the circadian clock system or not, since *Per2* has nonclock functions and may regulate the daily rhythms independently of the circadian clock system [4, 5].

Therefore, this short study aimed to clarify the precise role of the circadian clock system in the generation of daily rhythms in anaphylaxis. For this purpose, we examined the effects of SCN ablation on the time of day-dependent variations in PSA reaction since SCN ablation reliably eliminates the normal activity of the circadian clock system [4, 5].

2. Materials and Methods

2.1. Mice. Male 8–12-week-old ICR mice (Japan SLC, Tokyo, Japan) were bred under specific pathogen-free conditions and 12-hour light/12-hour dark conditions (L/D cycles; the light was turned on at 6:00 a.m., Zeitgeber time (ZT) 0, and the light was turned off at 6:00 p.m., ZT12) with *ad libitum* access to food and water, for at least 2 weeks. All animal experiments were approved by the Institutional Review Board of University of Yamanashi and Waseda University.

2.2. SCN Disruption. Bilateral thermal lesions of the SCN were obtained stereotactically (Narishige Co., Tokyo, Japan) under anaesthesia as described previously [7]. One month after the surgery, we selected animals with complete lesioning of the SCN after confirmation of arrhythmic general locomotor activity recorded with an infrared radiation sensor (F5B, Omron, Tokyo, Japan) and analyzed with CLOCK-LAB software (Actimetrics, Wilmette, IL, USA) as described previously [7]. A histological check of lesion sites was also performed after finishing the PSA experiments using Nissl staining.

2.3. Passive Systemic Anaphylactic (PSA) Reaction. A passive systemic anaphylactic (PSA) reaction was induced in mice as previously described with some modifications [8]. Briefly, mice were sensitized with an intravenous injection of 20 μg with mouse anti-TNP IgE in 0.2 mL PBS. The mice were intravenously challenged with 1000 μg DNP-BSA in 0.2 mL PBS 24 hours later. Their rectal temperature was measured with a digital thermometer (Shibaura Electronics, Tokyo, Japan) every 5 minutes from 20 minutes before DNP-BSA challenge and thereafter until 120 minutes after the start of the examination. The rectal temperature was also measured at 180 minutes after the start of the examination.

2.4. Serum MCP-1 (CCL2), IL-6, and Histamine Levels. Serum samples were collected from the mice at 10 minutes or 180 minutes after induction of PSA reactions. The amounts of MCP-1 (CCL2) and IL-6 in the serum at 180 minutes after induction of PSA reaction were measured using the mouse MCP-1 and IL-6 ELISA kits (R&D, Minneapolis, MN). The amounts of histamine in the serum at 10 minutes and 180 minutes after induction of PSA were measured using histamine EIA kit (Oxford Biomedical research, Inc., Oxford, MI, USA).

2.5. Serum IgE and Corticosterone Levels. Serum levels of total IgE and corticosterone were determined by using the mouse IgE ELISA kit (R&D, Minneapolis, MN, USA, or Morinaga Institute of Biological Science, Kanagawa, Japan) or AssayMax Corticosterone ELISA kit (AssayPro, Charles, MO, USA), respectively.

2.6. Statistics. Data were expressed as the mean ± S.D. Statistical analysis was performed using an unpaired Student's t-test to compare data in different groups. P value < 0.05 was considered to indicate a statistically significant difference.

3. Results and Discussion

In order to clarify the precise role of the circadian clock system in the generation of daily rhythms in systemic anaphylactic reaction, we examined the effects of SCN ablation on a time of day-dependent variations in passive systemic anaphylactic (PSA) reaction in mice, which is a representative model of anaphylaxis. We confirmed complete disruption of the SCN in mice by a histological check of lesion sites by Nissl staining after finishing all the experiments (Figure 1(a)) and by examining behavioral patterns (general locomotor activity with arrhythmicity) (Figure 1(b)).

The kinetics of PSA reaction was then compared in sham-operated mice and mice with disrupted SCN (Figure 2(a)). Sham-operated mice challenged with the antigen at 10:00 pm (ZT16) showed a smaller drop in the extent of rectal temperatures than the mice challenged at 10:00 am (ZT4). However, such a time of day-dependent variation in PSA reaction was absent in mice with disrupted SCN. Consistently, serum histamine, MCP-1 (CCL2), and IL-6 levels following the induction of the PSA reactions showed similar time of day-dependent variations to the PSA reactions in sham-operated mice, but the variations were absent in mice with disrupted SCN (Figures 2(b)–2(d)). The daily variations in serum corticosterone levels observed in sham-operated mice were also absent in mice with disrupted SCN (Figure 2(e)). In contrast, serum total IgE levels were comparable between sham-operated mice and mice with disrupted SCN (data not shown). These results indicated that disruption of the central clock SCN blunted a time of a day-dependent variation in PSA reaction in association with a loss of rhythmic secretion of corticosterone. These findings suggest that the circadian clock system may contribute to the generation of daily rhythms in anaphylaxis.

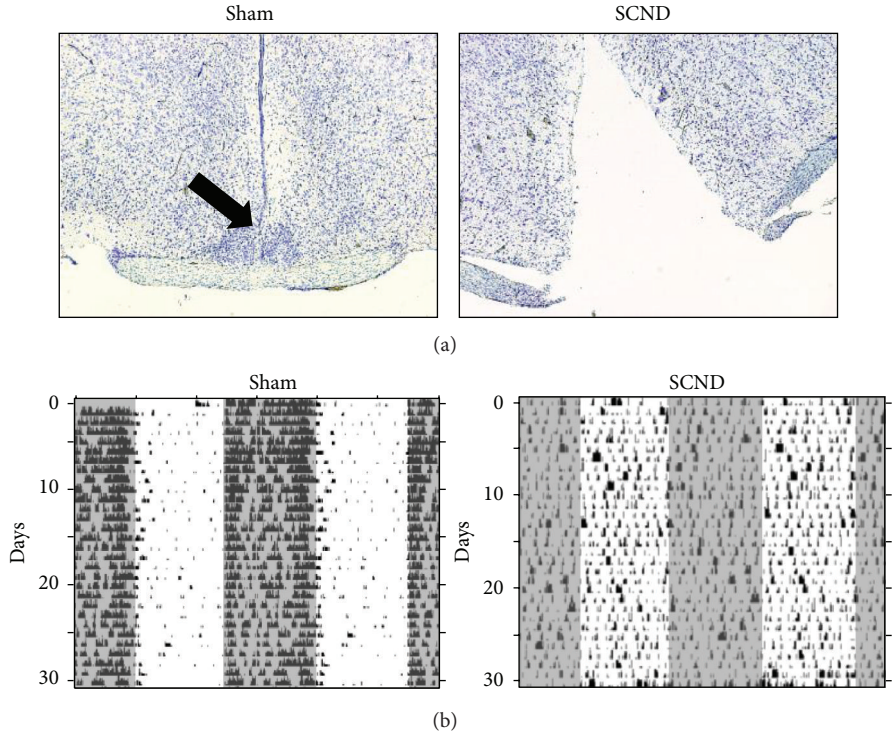

Figure 1: Histological and general locomotor analysis after induction of the bilateral electrolytic lesions of the SCN in mice. (a) The SCN of the mice was destroyed by bilateral electrolytic lesions and complete SCN destruction was ascertained by a postmortem histological analysis. Representative pictures of the staining in sham-operated mice (Sham) and mice with disrupted SCN (SCND) are shown. The arrow indicates the SCN area. (b) General locomotor activity of mice housed under LD 12 : 12 conditions was recorded and representative double-blot actograms of the drinking behavior of the mice during 30 days are shown. The black dots indicate the mouse locomotor activity. Horizontal open and solid bars indicate day and night, respectively. Please note that the drinking behavior (usually accompanied by food intake) became arrhythmic in mice with disrupted SCN (SCND).

Body temperature is under circadian control [4, 5]. However, it appears that the basal body temperatures at ZT4 and ZT16 before induction of PSA (0~20 minutes before the antigen challenge) were comparable in control sham-operated mice (Figure 2(a)). We speculate that this discrepancy might be caused, at least in part, by the experimental manipulation for measuring body temperature. Direct insertion of a digital thermometer into the rectum every 5 minutes likely affects mouse activity in the resting phase and might upregulate the body temperature. In contrast, a time of day-dependent variation in PSA reaction was clearly observed at ZT4 and ZT16 in control sham-operated mice (Figure 2(a)). Thus, it appears that the extent of a time of day-dependent systemic mast cell responses overrides the possible effects of the experimental manipulation on body temperature.

The surgery for SCN ablation may destroy other important nuclei located in this brain area such as temperature center and corticosterone releasing hormone (CRH) neurons. We observed that body temperatures at 180 minutes after the examination were comparable between sham-operated mice and mice with disrupted SCN (Figure 2(a)), suggesting that the brain area for temperature regulation was functional in mice with disrupted SCN. We also observed that basal levels of serum corticosterone were comparable between sham-operated mice and mice with disrupted SCN (Figure 2(a)), suggesting that the brain area for CRH regulation was functional in mice with disrupted SCN (Figure 2(e)). These findings may support the specificity of the surgery to the SCN although we cannot completely exclude some of the nonspecific influences of the SCN ablation on the current results.

It remains to be determined precisely how the dysfunction of the circadian clock (disruption of the SCN) affects the time of day-dependent variation in PSA reaction. We have previously shown that daily rhythms observed in passive cutaneous anaphylactic (PCA) reaction were absent in adrenalectomized mice [6], suggesting that humoral factors from the adrenal gland are necessary to maintain the daily rhythms. The central SCN pacemaker primarily governs the daily rhythmic secretions of humoral factors such as corticosterone from the adrenal gland [9]. Therefore, the SCN may control the time of day-dependent variation in PSA (and also PCA) reaction via regulation of the rhythmic secretion of humoral factors (possibly corticosterone) from the adrenal gland. The current findings that the time of day-dependent variation in PSA reaction in sham-operated mice showed an inverse association with the serum corticosterone levels and the extent of PSA reaction was severe in mice with disrupted SCN in association with low serum corticosterone levels may support this hypothesis.

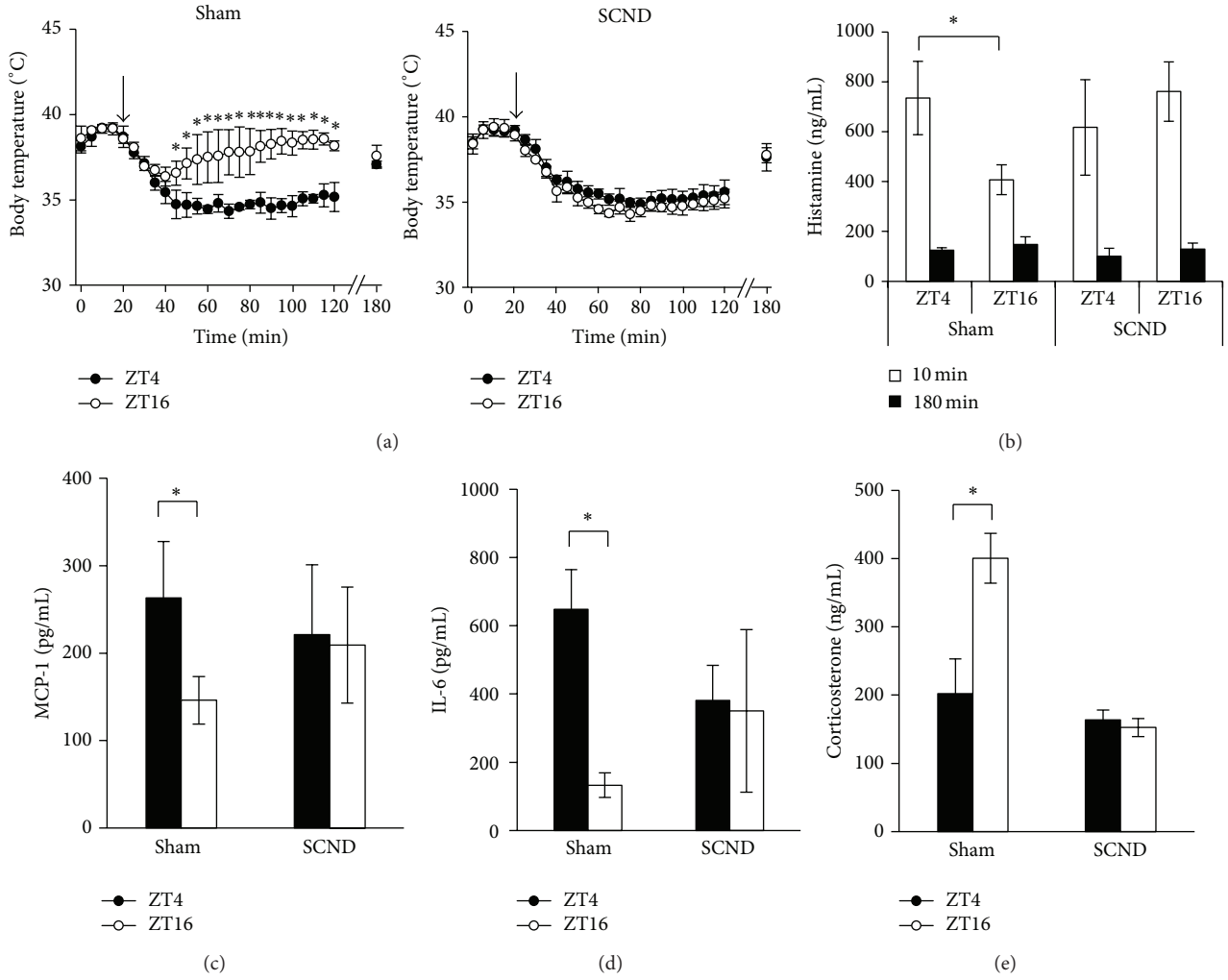

Figure 2: The SCN ablation blunts a time of day-dependent variation in PSA reaction. (a) Changes in rectal temperature over time after the allergen challenge at ZT4 (10:00 a.m.) and ZT16 (10:00 p.m.) in sham-operated mice (Sham) and mice with disrupted SCN (SCND) ($n = 4$). The arrows indicate the time of antigen challenge (b)–(d). The serum histamine (b), MCP-1 (CCL2) (c), and IL-6 (d) levels following induction of PSA reactions at ZT4 and ZT16 in sham-operated mice or mice with SCN disruption ($n = 4$ per group). Concentrations of histamine were measured using serum samples collected at 10 and 180 minutes after induction of PSA reaction at ZT4 or ZT10. Concentrations of MCP-1 and IL-6 were measured using serum samples collected at 180 minutes after induction of PSA reaction at ZT4 or ZT16. (e) The serum corticosterone levels at ZT4 and ZT16 in sham-operated mice and mice with SCN disruption ($n = 4$ per group). $^*P < 0.05$. Similar results were obtained in at least two independent experiments.

In summary, the current findings provide additional support for the notion that the circadian clock system times IgE-mediated systemic anaphylactic reaction [6]. The findings may provide a novel insight into the pathophysiology of anaphylaxis and also implicate that environmental and intrinsic factors disrupting the normal activity of the circadian clock system, such as sleep disturbance and mental stress [5, 6, 10], might predispose some types of allergic patients (e.g., patients with drug and food allergy) to severe anaphylaxis.

4. Conclusions

The circadian clock may be an important regulator of anaphylaxis, which will provide a novel insight into the pathophysiology of the disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yuki Nakamura, Kayoko Ishimaru, and Yu Tahara contributed equally to this work.

Acknowledgments

The authors thank Ms. Tomoko Tohno, Yuko Ohnuma, and Mutsuko Hara for their valuable general assistance. This

study was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 614529, 4 pages
http://dx.doi.org/10.1155/2014/614529

Review Article

Temporal Regulation of Cytokines by the Circadian Clock

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Received 4 February 2014; Accepted 19 March 2014; Published 6 April 2014

Academic Editor: Mizuko Mamura

Several parameters of the immune system exhibit oscillations with a period of approximately 24 hours that refers to "circadian rhythms." Such daily variations in host immune system status might evolve to maximize immune reactions at times when encounters with pathogens are most likely to occur. However, the mechanisms behind circadian immunity have not been fully understood. Recent studies reveal that the internal time keeping system "circadian clock" plays a key role in driving the daily rhythms evident in the immune system. Importantly, several studies unveil molecular mechanisms of how certain clock proteins (e.g., BMAL1 and CLOCK) temporally regulate expression of cytokines. Since cytokines are crucial mediators for shaping immune responses, this review mainly summarizes the new knowledge that highlights an emerging role of the circadian clock as a novel regulator of cytokines. A greater understanding of circadian regulation of cytokines will be important to exploit new strategies to protect host against infection by efficient cytokine induction or to treat autoimmunity and allergy by ameliorating excessive activity of cytokines.

1. Introduction

Several parameters of the immune system exhibit "circadian rhythms" which refer to daily oscillations with a period of approximately 24 hours [1, 2]. For instance, the number of specific immune cells (e.g., monocytes, neutrophils, and lymphocytes) in circulation and plasma levels of cytokines (e.g., TNF-α and IL-6) show ~24-hour daily rhythm that is translated into variations in acute response to infection [1, 2]. However, it remains poorly understood how such circadian immunity occurs. Recent studies reveal that the internal time-keeping system "circadian clock" is responsible for driving the circadian rhythms evident in the immune system. This review briefly summarizes new studies that provide mechanistic insights into how the immune system is under tight control of the circadian clock. In particular, this review puts emphasis on temporal regulation of cytokines by the circadian clock because cytokines play a central role in shaping immune response as crucial mediators for intercellular communication among immune and nonimmune cells. Better understanding of an emerging role of the circadian clock as a novel regulator of cytokines will be greatly helpful to improve timing of conventional treatment and to exploit new drug targets for immune-related diseases such as infection, autoimmunity, and allergy.

2. What Is the Circadian Clock?

The circadian clock drives the daily rhythms in behavior and physiology (e.g., sleep-wake cycles, body temperature, blood pressure, and hormonal secretions) that enable organisms to keep track of the time of day according to daily changes in light intensity [3, 4]. Mammalian circadian clock consists of the central oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus and peripheral oscillators virtually present in all cell types [3, 4]. Light activates a specific group of photoreceptors in the retina connected to the central SCN clock which entrains peripheral circadian clock via neural and endocrine pathways. The molecular mechanisms of rhythm generation are cell autonomous, highly conserved in the SCN and peripheral cells, and created and maintained by transcriptional-translational feedback loops that consisted of several "clock genes" and their protein products. Briefly, two transcription factors, CLOCK and BMAL1, activate the transcription of the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. The *Per* and *Cry* proteins in turn inhibit their own

expression by repressing CLOCK/BMAL1 activity. This negative feedback loop, with additional posttranscriptional modifications, generates ~24-hour oscillations of the clock protein levels and activity, which are translated into periodic changes of a variety of clock-controlled genes (CCGs) involved in important biological processes, including immune responses.

3. Circadian Regulation of Immunity

It has been well documented that many parameters in the immune system exhibit circadian rhythms [1, 2]. These include the number of circulating hematopoietic cells and the levels of cytokines. Not only the components of the immune system but also the immune functions, such as leukocyte migration/trafficking, and innate immune response also show circadian rhythms [1, 2]. However, biological basis behind those observations has been unclear. Recent excellent studies have provided compelling molecular evidence that the circadian clock is responsible for the temporal activities in the immune system [5–8].

Scheiermann et al. have recently reported that the central SCN clock drives circadian rhythms in the expression of adhesion molecules (e.g., ICAM-1 and VCAM-1) on endothelial cells or chemokines/chemokine receptors (e.g., CCL2 and CXCR4) in tissue or leukocytes, which contributes to a time of day-dependent recruitment of leukocytes into the tissues such as the bone marrow and muscle [9]. The circadian activity of sympathetic nervous system driven by the central SCN clock likely regulates the expression of adhesion molecules or chemokine/chemokine receptors in a temporal manner, thereby controlling leukocyte migration.

Nguyen et al. suggest another regulatory mechanism for leukocyte migration by the circadian clock [10]. They found that frequency of $Ly6C^{high}$ inflammatory monocytes (an innate immune cell type for the first line of defense against pathogens) in blood, spleen, and bone marrow exhibited circadian oscillations, which corresponded to the diurnal variations in recruitment of the cells into the sites of inflammation. They suggested that CLOCK/BMAL1 heterodimer negatively regulated expression of CCL2 in monocytes, which likely contributed to the circadian oscillations of $Ly6C^{high}$ inflammatory monocytes. In contrast to the study by Scheiermann et al. [9], this study therefore puts more emphasis on an important role of peripheral immune cell clock in leukocyte migration.

Silver et al. have provided molecular evidence how the circadian clock controls innate immune response [11]. They found that CLOCK/BMAL1 heterodimer bound to the promoter of Toll-like receptor (*Tlr9*) and promoted the expression and function in a circadian manner. Consistent with the findings, they showed that mice immunized at the time of enhanced TLR9 responsiveness presented strong innate immune reactions with an improved adaptive immune response.

Most recently, T cell development has been surprisingly shown to be regulated by the circadian clock proteins. Yu et al. reported that CLOCK/BMAL1-mediatd REV-ERBα induction inhibited expression of NF-IL3, a suppressor of Th17 cell development, thereby selectively influencing Th17 cell development [12]. Interestingly, circadian disruption by chronic light-cycle perturbations increased Th17 frequency in mouse intestine and worsened dextran-sodium sulfate (DSS)-induced colitis in mice [12]. Several other important immune functions have been also shown to be regulated by the circadian clock and the reader is encouraged to consult with excellent reviews for detailed overview of circadian immunity in many aspects [5–8].

4. Circadian Clock Proteins Temporally Gate Inflammatory Cytokine Expression

Cytokines are essential to shape immune responses and are indispensable for self-defense against infection. On the other hand, once the cytokine network goes out of control and an excessive amount of cytokines are produced, serious damage, such as sepsis or autoimmunity and allergy, occurs in the host animals. Therefore, expression of cytokines should be tightly regulated to maintain homeostasis of the immune system and, indeed, cytokine expression is regulated at multiple levels. Recent excellent studies reveal that the circadian clock is also an important regulator of cytokines [5–8]. Because circadian regulation of cytokines is extensively studied in *in vitro* and *in vivo* models of endotoxin LPS-induced inflammatory responses in innate immune cells, this review summarizes such studies to describe how the circadian clock regulates cytokine expression at the molecular levels.

Keller et al. reported that mouse macrophages derived from spleen, lymph nodes, and peritoneal cavity contain intrinsic circadian clockworks that operate autonomously as well as other innate and acquired immune cells [13]. Macrophages isolated from mouse spleen stimulated with LPS at different time points display circadian rhythms in TNF-α and IL-6 secretion and the cytokine secretion rhythms persist in constant *in vitro* culture conditions, suggesting that macrophage-intrinsic circadian clock may govern these oscillations [13]. Interestingly, ~8% of the expressed genes in peritoneal macrophages show circadian modulation including the genes involved in LPS-immune response pathways such as MD-1 and CD180 [13], which may explain rhythmic secretion of the cytokines upon LPS challenge.

It has been known since pioneering work of the 1960s and 1970s that mice challenged with LPS showed significant circadian-dependent variation in magnitude of response including mortality and cytokine concentrations in serum. Gibbs et al. showed that the temporal variations in serum IL-6 following LPS challenge were absent in mice with specific deletion of BMAL1 in myeloid cells [14], suggesting that myeloid cell-intrinsic clockwork provides temporal gating of cytokine responses to LPS. BMAL1 activates transcription of the nuclear receptor REV-ERBα and REV-ERBα, which in turn inhibits BMAL1 transcription, forming an accessory feedback loop that stabilizes the circadian clock machinery [4]. Because peritoneal macrophages exhibit a profound temporal variation in transcriptional factor REV-ERBα (~20-fold differences across the day) and the temporal variation of REV-ERBα was completely suppressed in Bmal1-deficient

peritoneal macrophages, Gibbs et al. investigated whether REV-ERBα was a key molecule linking the circadian and inflammatory pathways. As expected, rhythmic immune responses to LPS were abolished in REV-ERBα-deficient mice, suggesting a link among BMAL1, REV-ERBα, and IL-6 production in macrophages upon LPS challenge [14]. The precise biochemical mechanisms remain to be determined.

Spengler et al. showed that the severity of LPS-induced inflammatory response in mice was correlated with the intensity of NF-κB activation and that NF-κB activation is, in fact, under circadian control based on experiments using NF-κB reporter mice [15]. They demonstrated that the core circadian protein clock bound to the p65 subunit of NF-κB, acetylated p65, and upregulated NF-κB-dependent transcriptional responses. Interestingly, clock acts as a positive regulator of NF-κB-responsive genes distinct from the transactivation of circadian genes via E-box elements that i, independent of its circadian function [15].

Narasimamurthy et al. reported another mechanism of how the circadian clock controls inflammatory cytokine expression [16]. They showed that *Cry*-deficient macrophages exhibited a marked increase in TNF-α and IL-6 protein secretions compared with wild-type macrophages, suggesting that *Cry*-deficient macrophages were hypersensitive to LPS stimulation. They also showed that *Cry* binds to adenyl cyclase, resulting in enhanced levels of cAMP and constitutive PKA activity and NF-κB activity in macrophages [16]. Because *Cry*-deficient macrophages constitutively upregulated TNF-α and IL-6 expression, an important implication of this study is that an arrhythmic clock system may be sufficient to increase the stress levels of cells leading to constant expression of inflammatory cytokines and causing a low-grade chronic inflammatory status.

Collectively, these findings provide molecular insights into how the circadian clock regulates daily variations in the induction of inflammatory cytokines upon a powerful challenge of LPS and how disruption of the clockwork affects the cytokine responses. In particular, these findings clearly demonstrate that certain clock proteins interact with important regulatory pathways for cytokine expression. The results may explain the daily fluctuations in susceptibility to various pathogens to the immune system.

5. Circadian Regulation of Cytokines Involved in Inflammatory Diseases

Many chronic inflammatory diseases show circadian rhythms in disease severity or symptoms [8]. However, it remains largely unclear whether the circadian clock is attributed to such circadian pathophysiology. Rheumatoid arthritis (RA) is one of the representative chronic inflammatory diseases with circadian pathophysiology. Patients with RA exhibit bad joint inflammation, pain, and stiffness in the morning hours, which is associated with increased inflammatory cytokine production. Recently, Hashiramoto et al. showed the link between RA and the circadian clock for the first time. They demonstrated that *Cry*-deficient mice exhibited enhanced arthritis associated with upregulation of inflammatory cytokines including TNF-α [17]. Their data are largely consistent with the findings that *Cry* may be a suppressor for inflammatory cytokine expression [16].

Many symptoms in allergic diseases exhibit prominent ~24-hour variation [18, 19]. For instance, in most allergic rhinitis patients, symptoms worsen overnight or early in the morning "morning attack" and often compromise night-time sleep, which results in poor daytime quality of life [20]. Such phenomena were actually recognized long before the birth of chronobiology. For example, Caelius (4th or 5th century AD) noted the frequent nocturnal occurrence of asthma attacks [21]. However, the precise mechanisms remain enigmatic.

Nakamura et al. have recently provided evidence that the circadian clock temporally controls IgE/mast cell-mediated allergic reaction *in vivo* [22]. They showed the time of day-dependent variation observed in IgE-mediated immediate type allergic reaction in the skin of wild-type mice (passive cutaneous anaphylactic [PCA] reaction) to be absent in *Per*2-mutant mice (mPer2$^{m/m}$ mice) [23]. Most recently, they have also shown that the circadian clock present in mast cells primarily contributes to the generation of the daily rhythms observed in PCA reaction by temporally regulating FcεRI (the high affinity IgE receptor) expression and signaling [24]. Thus, allergic reaction also appears to be under tight control of the circadian clock. Future studies will ensure that many other chronic inflammatory diseases with circadian pathophysiology are under control of the circadian clock.

6. Conclusions

Recent strong molecular evidence shows an involvement of certain clock proteins in the circadian gating of immune responses. Importantly, new studies demonstrate direct interaction of certain clock proteins with regulatory pathways for cytokine expression in immune cells, which may contribute to shaping circadian immunity such as temporal variations observed in leukocyte trafficking, T cell development, innate immune defense against pathogens, and autoimmunity/allergy. Interestingly, disruption of the circadian clock appears to aggregate chronic inflammatory diseases such as RA via aberrant expression of inflammatory cytokines [6, 7, 17]. The findings may implicate that increased prevalence of human inflammatory diseases including autoimmunity and allergy in industrialized countries may be linked to modern life style chronically disrupting the circadian clock such as night shift work or jet lags. Thus, it will be a significant challenge in future how new basic studies on circadian control of immunity, especially on circadian regulation of cytokines, will be translated into prevention and treatment of infection, autoimmunity, and allergy.

Conflict of Interests

The author declares no financial or commercial conflict of interests.

Funding

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Acknowledgments

The author thanks Ms. Tomoko Tohno, Yuko Ohnuma, and Mutsuko Hara for their valuable general assistance.

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Journal of Immunology Research
Volume 2014, Article ID 945829, 10 pages
http://dx.doi.org/10.1155/2014/945829

Clinical Study

The Impact of Two Different Transfusion Strategies on Patient Immune Response during Major Abdominal Surgery: A Preliminary Report

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Received 6 January 2014; Revised 13 March 2014; Accepted 13 March 2014; Published 3 April 2014

Academic Editor: Jana Barlic-Dicen

Blood transfusion is associated with well-known risks. We investigated the difference between a restrictive versus a liberal transfusion strategy on the immune response, as expressed by the production of inflammatory mediators, in patients subjected to major abdominal surgery procedures. Fifty-eight patients undergoing major abdominal surgery were randomized preoperatively to either a restrictive transfusion protocol or a liberal transfusion protocol (with transfusion if hemoglobin dropped below 7.7 g dL^{-1} or 9.9 g dL^{-1}, respectively). In a subgroup of 20 patients randomly selected from the original allocation groups, blood was sampled for measurement of IL-6, IL-10, and TNFα. Postoperative levels of IL-10 were higher in the liberal transfusion group on the first postoperative day (49.82 ± 29.07 vs. 15.83 ± 13.22 pg mL^{-1}, $P < 0.05$). Peak postoperative IL-10 levels correlated with the units of blood transfused as well as the mean duration of storage and the storage time of the oldest unit transfused ($r^2 = 0.38$, $P = 0.032$, $r^2 = 0.52$, $P = 0.007$, and $r^2 = 0.68$, $P < 0.001$, respectively). IL-10 levels were elevated in patients with a more liberal red blood cell transfusion strategy. The strength of the association between anti-inflammatory IL-10 and transfusion variables indicates that IL-10 may be an important factor in transfusion-associated immunomodulation. This trial is registered under ClinicalTrials.gov Identifier: NCT02020525.

1. Introduction

Major abdominal surgery can often be complicated by massive hemorrhage with all the sequelae that profound anemia entails in this setting [1]. Therefore, blood transfusion is considered a cornerstone of perioperative care practice and is used to augment oxygen delivery in the hope of avoiding the deleterious effects of serious anemia and the resulting oxygen debt, especially in vulnerable patients [2].

On the other hand, the administration of blood products is associated with well-described adverse consequences. In particular, allogeneic red cell transfusions can potentially lead to the transmission of viral infections, febrile nonhemolytic transfusion reactions, or alloimmunization to human leukocyte antigens [3]. There is always a small but distinct possibility for bacterial contamination as well as for errors in blood administration. Furthermore, the prolonged storage of blood products may decrease the ability of the red cell to transport or deliver oxygen through an abnormal microcirculation [4–6]. There is also growing concern about limited supply and the escalating cost of blood transfusion.

More importantly, transfusion of blood products has been linked with the induction of clinically important immune suppression, which may unfavorably affect the postoperative course by increasing tumor recurrence rate or the potential for serious nosocomial infections [7, 8]. The surgical trauma itself causes a systemic inflammatory response through activation of various cellular and humoral cascade systems [9]. Whenever blood transfusion is needed, a secondary inflammatory insult might ensue, which enhances the initial inflammatory response evoked by the surgical procedure [10, 11]. The mechanisms involved in the immunomodulatory effect

of allogeneic blood transfusion have not been elucidated yet, but it has been suggested that these adverse effects may be mediated by white blood cells present in transfused cellular blood components and the generation of inflammatory mediators [12]. Transfusion-associated immunomodulation (TRIM) has also been associated with the duration of storage of blood components [5, 13, 14] and the impairment in the balance between proinflammatory (tumor necrosis factor (TNFα), interleukin-1 (IL-1), IL-6), and anti-inflammatory circulating cytokines (IL-4, IL-10) [15, 16].

For all these reasons, there is a trend leading to reassessment of transfusion strategies and over the recent years there have been recommendations that physicians lower the trigger of hemoglobin (Hb) level at which patients are transfused. However, the level of Hb that most accurately predicts the need for blood transfusion has been widely debated and transfusion practices still remain highly variable and controversial.

We have previously reported the results of the primary and secondary outcomes of a randomized study aiming to investigate the impact of a restrictive transfusion protocol on the magnitude of reduction in blood transfusion in a typically mixed general surgery population subjected to major abdominal surgery [17]. The main finding of that study was a reduction in red blood cell usage with the implementation of a restrictive transfusion regimen. Notably, this was achieved without adversely affecting clinical outcome in the population studied.

The aim of this secondary analysis performed on a subgroup of 20 patients from the original study was to determine whether there are any differences in the postoperative immunologic response, as expressed by the production of inflammatory mediators, between a restrictive approach to red cell transfusion and a more liberal strategy.

2. Methods

2.1. Study Subjects. The study was approved by the Institutional Review Board of Areteion University Hospital. The study protocol has been presented in detail previously [17]. Patients scheduled for elective upper major abdominal surgery were enrolled in the study after providing written informed consent. Exclusion criteria were a history of bleeding diathesis and refusal of transfusions for religious reasons or a history of active ischemic heart disease (unstable angina or myocardial infarction within 6 months preceding the scheduled operation). Moreover, patients with preexisting infectious or autoimmune diseases were excluded from participation as well as those patients having used corticosteroids or immunosuppressive drugs within 6 months.

Patients were randomized preoperatively to one of two intraoperative and postoperative transfusion strategies. Sealed opaque envelopes containing odd and even numbers were chosen at random for patient assignment. Patients assigned to the liberal strategy were transfused when their hemoglobin concentration fell below 9.9 g dL^{-1}, aiming at maintaining hemoglobin at or above 10 g dL^{-1}. Patients allocated to the restrictive transfusion strategy were transfused only when their hemoglobin concentration decreased below 7.7 g d dL^{-1} and were then maintained at hemoglobin concentrations between 7.7 and 9.9 g d dL^{-1}.

2.2. Transfusion Management. All patients were operated under using the same anesthetic protocol, while antibiotic prophylaxis and postoperative analgesia were also standardized. Transfusion guidelines and group assignment were followed both intraoperatively and postoperatively. Both the surgical team and anesthesiologists responsible for the patient were informed as to the allocation group. Intraoperative transfusions were supervised by the anesthesiologist in charge of the protocol and postoperative transfusions by both the surgeon and anesthesiologist in charge. Ward personnel were informed about transfusion strategy assignment to ensure compliance with the protocol with the aim to treat transfusion trigger deviations as protocol violations. Moreover, adherence to the transfusion protocol was ensured by blood transfusion being prescribed only by the research team involved in the study. All transfusions were nonleukodepleted packed red blood cells (RBCs) stored in citrate-phosphate dextrose adenine-1 (CPDA-1). The maximum duration of storage of erythrocyte units is 42 days according to policies followed by blood banks across the world [18]. The date of collection of each unit transfused was retrieved from blood bank records and the length of storage of each unit transfused between the date of collection and the date of transfusion was calculated. Transfusions were administered one unit at a time and hemoglobin concentration was measured in all study patients with the HemoCue 201 DM device (HemoCue, Inc., Cypress, CA, USA) after each red blood cell unit had been transfused. Compliance to the transfusion protocol was monitored by daily measurements of hemoglobin concentration in each patient.

2.3. Study Endpoints and Postoperative Follow-Up. Primary outcome measure of the original study was red blood cell usage, as expressed by the number of units transfused per patient as well as the difference in the incidence of blood transfusions between the two randomization groups [17]. In this secondary analysis performed on a subgroup of 20 patients randomly selected from the original allocation groups, blood was sampled for measurement of IL-6, IL-10, and TNFα preoperatively, 6 hours, one day, and three days postoperatively. Time of mobilization, time of first liquid and solid food intake, and length of postoperative hospital stay were also recorded for each patient. Additionally, patients were followed up on a daily basis until hospital discharge during which time the incidence of all postoperative infectious complications was recorded. Finally, two variables were used to study the effect of the length of storage of the RBC units transfused to each patient: (1) the mean length of storage of all RBC units transfused and (2) the length of storage of the oldest unit transfused per patient.

2.4. Cytokine Analysis. In a subgroup of patients (10 patients randomly selected from each transfusion policy allocation group) IL-6, IL-10, and TNFα were measured. Peripheral

venous blood was drawn at the following time points: preoperatively, 6 hours, one day, and three days postoperatively. All samples were collected in sterile tubes (Vacutainer, Becton-Dickinson, Heidelberg, Germany) and were immediately centrifuged and the supernatant was stored at −60°C until assay. Quantitative determination of cytokine levels was performed using commercially available sensitive immunoassay kits (Quantikine HS human IL-6, Quantikine HS IL-10, and Quantikine HS human TNFα for IL-6, IL-10, and TNFα, resp.) (R&D Systems Inc. 614 McKinley Place NE, MN, USA), according to the recommendations of the manufacturer. Detection sensitivity was 0.039 pg mL^{-1} for IL-6, 3.9 pg mL^{-1} for IL-10, and 0.106 pg mL^{-1} for TNFα. The coefficient of variability of the method was 6.5–9.6% for IL-6, 4.3–7.5% for IL-10, and 5.3–16.7% for TNFα. All assays were performed in duplicate and averaged data were used in the subsequent analysis.

2.5. Statistics. Power calculation and estimation of sample size were based on the primary outcome measure of the original trial and have previously been described in detail [17]. Variables were tested for normality of distributions with the Kolmogorov-Smirnov test. Comparisons of numeric data between the two groups were performed with the unpaired t-test or the Wilcoxon rank sum test for independent samples, depending on whether the variables followed a normal or nonnormal distribution. The chi-square test or Fisher's exact test, as appropriate, was used for comparisons of categorical data. Correlation between data was tested by using the Pearson product moment correlation coefficient test. Stepwise multiple linear regression analysis was performed in order to adjust for the effect of confounding and to investigate the independent predictive value of variables. The postoperative changes in Hb levels as well as serial changes in IL-6, Il-10, and TNFα levels were analyzed with two-factor mixed design analysis of variance with repeated measures for one factor (time). The two factors were the subject group and time and the Student-Newman-Keuls method was used *post hoc* for pairwise multiple comparisons. Results are expressed as mean SD or as median (25th–75th percentiles) depending on normality of distributions. A value of $P < 0.05$ was considered as statistically significant. Statistical analysis was performed by the use of SPSS for Windows v.16.0 statistical software (SPSS Inc., Chicago, Il, USA).

3. Results

The 20 patients randomly selected from the two transfusion allocation groups did not differ significantly in demographic characteristics, namely, age, weight, height, sex, American Society of Anesthesiologists (ASA) distribution, and the type of surgical procedures performed. The postoperative serial changes in the circulating levels of IL-6, IL-10, and TNFα in these two subgroups of patients are summarized in Figure 1. IL-6 was distinctly higher from baseline at all time points in both subgroups. No intergroup differences were demonstrated for IL-6 at any time point. IL-10 also exhibited a postoperative increase as compared to baseline in the two transfusion policy groups, which was obvious 6 and 24 hours postoperatively, with a subsequent decline to near baseline ranges at the end of the observation period. However, postoperative systemic induction of IL-10 was significantly exaggerated in patients subjected to a higher volume of transfusion ($P < 0.05$ for intergroup comparison 24 hours postoperatively). Postoperative concentrations of TNFα were not significantly different from baseline in either subgroup. TNFα levels were lower on the third postoperative day in the liberal transfusion group as compared to the restrictive group ($P < 0.05$ for intergroup comparison).

Peak postoperative IL-10 levels were found to correlate significantly with the units of blood transfused ($r^2 = 0.38$, $P = 0.032$) (Figure 2). Strong correlations between peak postoperative IL-10 values and the mean duration of storage of blood transfused (in days) (Figure 3) as well as the storage time (in days) of the oldest unit transfused (Figure 4) were also demonstrated ($r^2 = 0.52$, $P = 0.007$ and $r^2 = 0.68$, $P < 0.001$, resp.). No correlations for the other two mediators were demonstrated. Furthermore, we entered the units of blood transfused, the mean age of the blood transfused, and the storage time of the oldest unit transfused, which were associated with peak IL-10 values by univariable analysis, in a multivariable stepwise linear regression analysis model. Multivariable regression analysis showed that all three factors were independent variables significantly associated with peak postoperative IL-10 levels ($P = 0.04$, $P = 0.02$, and $P = 0.009$, resp.).

Outcome data of the 20 patients who participated in this secondary subgroup analysis are presented in Table 1. Overall RBC usage (units/patient) in the restrictive strategy group was 0 [0, 2] (median [IQ range]) as compared to 1.5 [1, 3] in the liberal strategy group ($P = 0.037$). Average postoperative hemoglobin concentration was 9.6 ± 1.1 g dL^{-1} in the restrictive group versus 10.7 ± 1.0 g dL^{-1} in the liberal group ($P = 0.004$). The duration of storage of transfused blood (in days) was shorter in the restrictive strategy group than in the liberal strategy group (21.7 ± 10.9 versus 28.5 ± 6.3, $P = 0.044$). The two groups did not differ in the time of mobilization, time of first liquid, and solid food intake as well as in the length of hospital stay ($P = 0.414$, 0.550, 0.139, and 0.643, resp.). Similarly, there was no difference in the rate of infectious complications between the two transfusion allocation groups. Finally, there was a trend for higher peak values of IL-10 in the seven patients who developed postoperative complications, although not statistically significant ($P = 0.09$) (Figure 5).

4. Discussion

The main finding of this secondary post hoc analysis was the higher level of IL-10 24 hours postoperatively in the group that received more blood transfusions intraoperatively and postoperatively in comparison to the restrictive transfusion group. Additionally, peak postoperative IL-10 levels were found to correlate with the units of blood transfused as well as the mean duration of storage and the storage time of the oldest unit transfused. In both transfusion allocation groups,

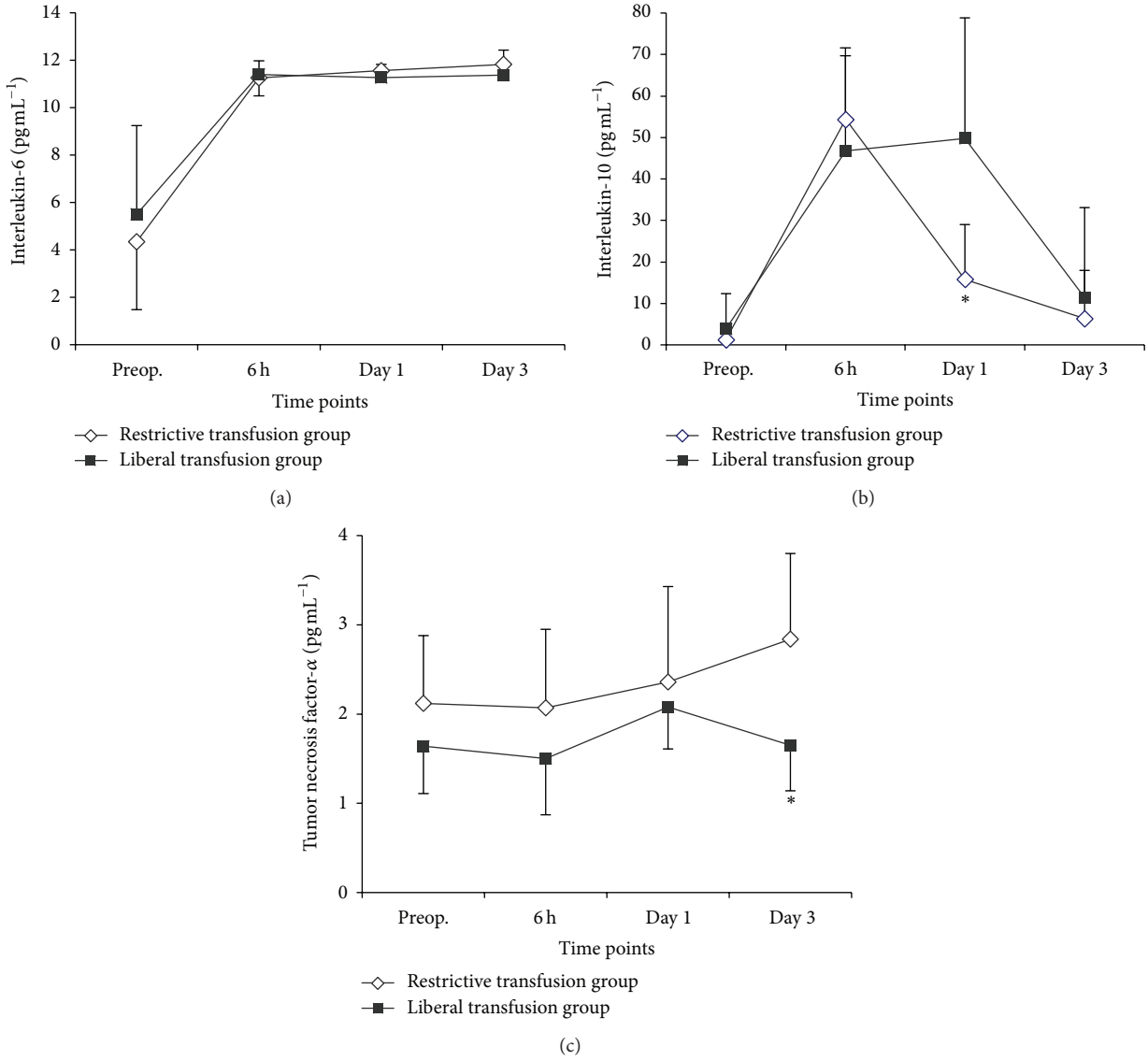

Figure 1: (a) Serial changes in perioperative IL-6 levels. Data are presented as mean ± SD. No intergroup differences were demonstrated. ($P < 0.001$, effect of time; $P = 0.462$, group by time interaction). (b) Serial changes in perioperative IL-10 levels. Data are presented as mean ± SD. Postoperative systemic induction of IL-10 was significantly exaggerated in the liberal transfusion group 24 h postoperatively. ($^{*}P < 0.05$ for intergroup comparison; $P < 0.001$, effect of time; $P < 0.001$, group by time interaction). (c) Serial changes in perioperative TNFα levels. Data are presented as mean ± SD. There was a difference between the two groups on the third postoperative day. ($^{*}P < 0.05$ for intergroup comparison; $P = 0.842$, effect of time; $P = 0.029$, group by time interaction).

there was a postoperative increase in the concentration of IL-6 and IL-10 in comparison to baseline.

RBC transfusion can be life-saving in severe hemorrhage, following major trauma or as a complication of major surgery and its benefits in these indications are undisputed. However, allogeneic blood products are a scarce and increasingly expensive resource, which is not risk-free. Among other risks, allogeneic blood transfusion has been incriminated in transfusion-associated immunomodulation, with initiation of a secondary inflammatory response enhancing the inflammatory insult evoked by the surgical procedure. The postoperative increase in the concentration of inflammatory cytokines demonstrated in our secondary analysis is in accordance with other studies which have shown ample release of various inflammatory mediators after surgery [9, 19]. In fact, it has been shown that the surgical trauma induces a profound inflammatory response through activation of complex cascade systems among which cytokines seem to play an important role in the acute phase. The release of these mediators is considered protective at least initially, since it aims at promoting healing of damaged tissues. However, the exaggerated and prolonged postoperative cytokine responses as well as any imbalance between proinflammatory and counterregulatory influences may lead to damage of otherwise healthy tissues and lead to the development of multiorgan failure and increased mortality [9, 20]. TNF-α is

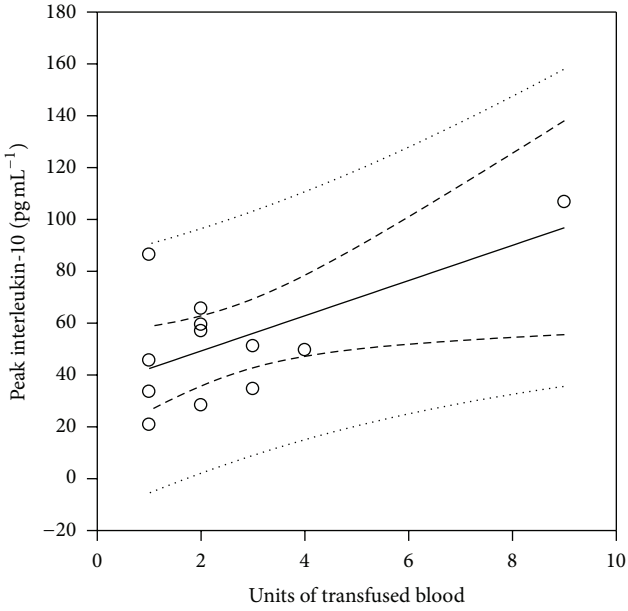

Figure 2: Scatter plot diagram of peak postoperative IL-10 values versus the number of units transfused, depicting a significant correlation ($r^2 = 0.38$, $P = 0.032$).

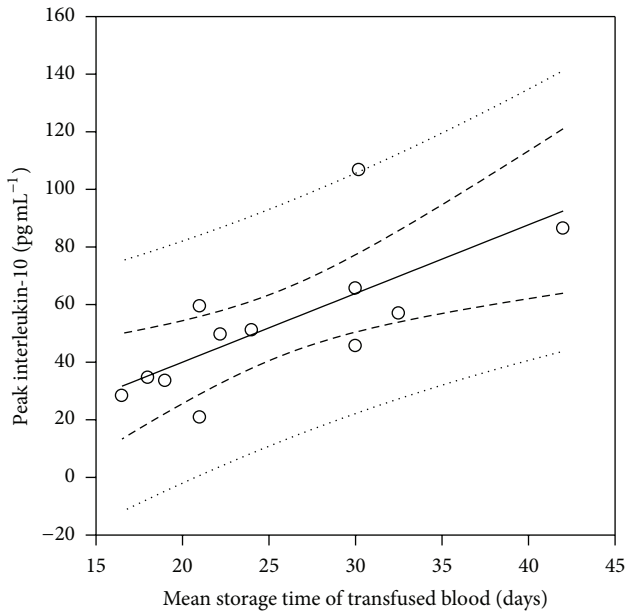

Figure 3: Scatter plot diagram of peak postoperative IL-10 values versus the mean duration of storage of transfused blood (in days). The storage time of transfused blood demonstrated a strong correlation to peak IL-10 values ($r^2 = 0.52$, $P = 0.007$).

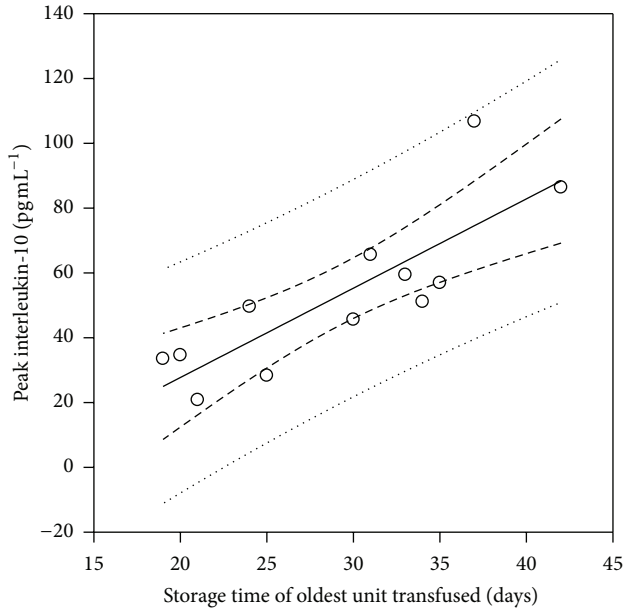

Figure 4: Scatter plot diagram of peak postoperative IL-10 values versus the duration of storage (in days) of the oldest unit of blood transfused. A strong correlation between the storage time of the oldest unit transfused and peak IL-10 values was demonstrated ($r^2 = 0.68$, $P < 0.001$).

one of the first bioactive substances released and although it is not always detectable in the early phase following trauma probably due to its short half-life [9], it mediates the release of another proinflammatory substance, IL-6 [21–23]. IL-6 is released in response to a variety of stimuli, including major surgery and thermal injury [24]. It is a reliable marker of tissue injury, it is almost constantly detected postoperatively, and its systemic levels reflect the severity of the surgical impact [25–27].

It is not always easy to decide whether the postoperative cytokine surge is causally related to the extent of blood transfusion or to the circumstances that preceded or necessitated it. Thus, distinguishing the immunomodulatory effects of surgery from the effects of transfusion can be quite difficult. In our study, however, IL-6 showed similar plasma concentrations at equivalent time points postoperatively. The lack of differentiation between the two groups might imply that the surgical impact itself is predominantly responsible for IL-6 release and that the role of blood transfusion may be less definitive for IL-6 fluctuations postoperatively [9, 19, 28]. In contrast, although the initial pattern of IL-10 release was similar in both patient groups, there was a clear differentiation 24 h postoperatively in IL-10 levels between the two groups. By that time, IL-10 levels were significantly elevated in patients with excessive red blood cell supply. The observed difference in the postoperative time course and magnitude of IL-10 release may be largely attributable to the different transfusion therapy *per se*.

Although perioperative blood transfusion is thought to synergistically exaggerate the surgery-evoked cytokine response, it seems to induce a higher immunosuppressant than a proinflammatory effect. In clinical investigations, significant immunosuppression as a result of allogeneic blood transfusion has been suggested to contribute to the high recurrence rate of malignancies and to transplant rejection episodes [29]. The balance between proinflammatory and inflammatory cytokines is crucial for the host immune

Table 1: Outcome data in the 20 patients of the restrictive and liberal transfusion group who were sampled for perioperative cytokines.

Parameter	Restrictive strategy group ($n = 10$)	Liberal strategy group ($n = 10$)	P value
RBC usage (units/patient)	0 [0, 2]	1.5 [1, 3]	0.037
Average postoperative Hb (g dL^{-1})	9.6 ± 1.1	10.7 ± 1.0	0.004
Duration of blood storage (days)	21.7 ± 10.9	28.5 ± 6.3	0.044
Time of mobilization (days)	2 [1, 2]	1 [1, 3]	0.414
Time of first liquid intake (days)	2 [2, 3]	2.5 [2, 3]	0.550
Time of first solid intake (days)	3 [2, 4]	5 [3–5]	0.139
Length of hospital stay (days)	7 [5, 7]	7 [5, 10]	0.643
Pulmonary complications	1	4	0.303
Intra-abdominal collection	0	1	1.000
Urinary infection	0	0	1.000
Wound infection	0	1	1.000

Values are mean ± SD for parametric numeric data, median [25th–75th percentiles] for nonparametric numeric data, and number (percentage) for categorical data; RBC: red blood cells; Hb: hemoglobin.

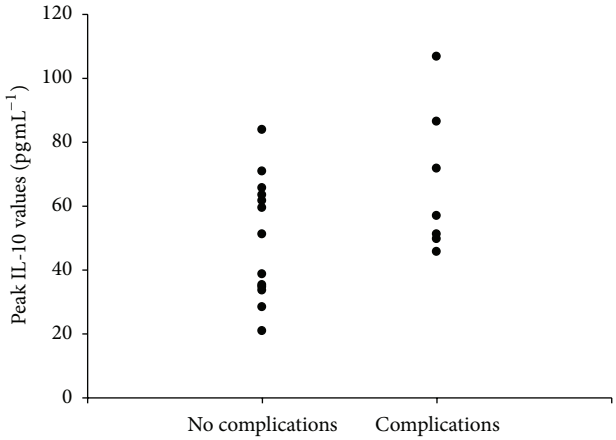

Figure 5: Scattergraph of peak postoperative IL-10 values in the seven patients who developed postoperative complications and in the 13 patients who did not. A trend for higher peak IL-10 values in the patients with complications was demonstrated ($P = 0.09$).

response and any derangement can lead to host defense failure [30] or enhance susceptibility to infectious complications [10, 11]. In fact, in the original randomized study, there was a tendency for an increased rate of respiratory infectious complications in the liberal transfusion group, although not statistically significant [17]. This trend was not observed in the subgroup analysis, obviously due to the low number of patients that were allocated to cytokine sampling. However, the trend for an increased rate of respiratory complications in the liberal transfusion group, as described in the original study, is consistent with literature reporting a dose-response relationship between the number of units transfused and the risk for postoperative infection [7, 28]. Both quantitative and qualitative immunologic alterations might predispose the recipient of a high blood transfusion volume to an increased risk for bacterial infections [7]. As already mentioned, blood transfusion has been shown to be associated with clinically important immunosuppression [10, 11], which may be mediated through the release or overexpression of IL-10. IL-10 is mainly considered anti-inflammatory and the predominance of anti-inflammation may lead to immunosuppression ("immunoparalysis"). IL-10 has been shown to downregulate a number of monocyte/macrophage actions and to prevent migration of polymorphonuclear leukocytes and eosinophils to sites of inflammation [15, 16, 31]. Additionally, high circulating levels of IL-10 impair leukocyte activation and degranulation [32]. IL-10 has also been suggested to play a role in downregulation and suppression of T-helper cell function [33, 34]. Immunosuppression mediated through IL-10 can increase mortality because it hampers the effective clearance of infectious agents in an experimental setting of bacterial pneumonia while inhibition of IL-10 bioactivity prolongs survival in a similar setting [35, 36]. Moreover, IL-10 predominance over proinflammatory mediators is correlated with poor patient survival after sepsis [37]. In our study, the possibility of a causal association between IL-10 and blood transfusion is further supported by the fact that, in this subanalysis, peak IL-10 values were found to correlate with the volume of transfused blood administered. The higher levels of IL-10, the time course of its release as well as in the greater incidence of postoperative respiratory complications in the liberal transfusion group in the original study, and the trend for higher peak values of IL-10 in the seven patients who developed postoperative complications in this subgroup analysis (although not statistically significant, probably due to the small number of patients sampled for cytokine measurements) might reflect the difference in transfusion policy between the two groups. Our results extrapolate data already shown in experimental studies to a clinical setting. Specifically, in an experimental study, allogeneic stored blood resulted in a significant TNF-α depression and IL-10 reduction when it was added to whole blood of a recipient and subjected to coculture, mimicking an *in vitro* model of blood transfusion [38]. Moreover, in a mice study, allogeneic blood transfusion led to a 5-fold increase in IL-10 production, which did not return to control levels before day 30 after transfusion

[39]. Finally, Mynster presented *in vitro* evidence of reduced responsiveness of innate immune cells along with an increase in IL-10 production after incubation of freshly donated blood with allogeneic stored red blood cells [40].

In our subanalysis, peak IL-10 values were also found to correlate with the storage time of blood units administered. The generation of inflammatory mediators is, to some extent, affected by storage duration due to degeneration of leukocytes with increased storage time. With the disintegration of leukocytes, leukocyte-derived and other biologic response modifiers accumulate extracellularly during storage in a time-dependent manner and may play a significant role in immunosuppression and tissue damage [41, 42]. Erythrocytes also undergo many corpuscular changes during storage and the accumulation of toxic factors in the red cell membrane might also contribute to storage time-dependent dysregulation of immunity [43]. Moreover, in RBCs stored for a long time, depleted levels of 2,3 diphosphoglycerate with the resulting leftward shift in the oxyhemoglobin dissociation curve and altered RBC rheology may impede regional blood flow and adversely affect oxygen delivery to tissues [4, 44–46]. These may be involved in storage time-dependent infectious side-effects in the recipient by predisposing to splanchnic ischemia. Whether factors associated with duration of storage induce the observed increase in IL-10 release in the liberal transfusion group has yet to be established. To the best of our knowledge, it is the first time in the literature that such a correlation has been demonstrated. In fact, the tendency we found for an increased rate of respiratory complications (albeit without statistical significance) in the group with the greater volume of blood transfusion in the original study might alternatively be attributed to IL-10-storage-time- dependent dysregulation of immunity, since this group received blood of older age. This finding is in accordance with other observational studies relating duration of storage with morbidity [41, 47–50]. We cannot of course rule out the possibility that confounding might have affected our results since, in the design of our original protocol, the distribution of length of storage of blood units among the two groups was not truly random; according to our results, patients who were exposed to a higher volume of blood received a greater proportion of RBC units stored for longer periods as compared to patients who had fewer RBC transfusions. This however could be related to the fact that large transfusion requirements increase the possibility of transfusing blood units with long storage time. Additionally, our hospital blood bank tends to release the oldest RBC units first, following policies adopted by most hospital transfusion services. Thus, it is more likely for patients requiring a higher number or erythrocytes to receive transfusion with older units. However, we believe that the strength of the association between IL-10 values and storage variables in our study might imply a direct relation between IL-10 and age of blood administered. Additionally, multivariate regression analysis showed that both volume and age of blood transfused were independently associated with IL-10 values. A reliable way to eliminate the effect of any confounding and to detect a more solid association between storage duration of transfused blood and complications would be to design trials randomizing patients to different lengths of storage of transfused units. Such randomization however might be ethically unacceptable and therefore conclusions can mostly be reached from observational studies.

In contrast to IL-10 and IL-6, postoperative systemic concentrations of TNFα were only slightly elevated. This is consistent with the literature and may have to do with the sensitivity of the detection method involved (resulting in small differences in mediator levels to go undetected) or may be due to rises occurring only transiently during surgery; recovering by the time blood was sampled after surgery [9, 21]. Studies have demonstrated the postoperative induction of soluble TNF receptors, which may bind and inactivate TNFα [51]. IL-10 has also been shown to downregulate the production of TNFα from human alveolar macrophages and peripheral blood monocytes [52, 53]. In fact, in our study, the slight decrease in TNFα levels observed on the third postoperative day in the liberal transfusion group followed the surge of IL-10, which shows that the time course and variation of TNFα may be additionally regulated by the presence of anti-inflammatory IL-10.

The major limitation of this secondary post hoc analysis is that cytokines were analyzed in only a subgroup of patients due to the high cost of the measurement kits and to hospital budget limitations. We however believe that our results are relevant and give some insight especially into the potential association of IL-10 and transfusion-related parameters. Another consideration is that nonleukoreduced blood was used for transfusion, which could have had an impact on the levels of mediators studied. Despite the fact that the mechanisms involved in the immunomodulatory effect of allogeneic blood transfusion have not been thoroughly elucidated yet, it has been suggested that the majority of these effects is mediated by the interaction of white blood cells (or their products) in transfused blood and anti-leukocyte antibodies in the recipient plasma [54–56]. It has also been shown that patients transfused with blood without prestorage leukocyte reduction might present lymphocyte count alterations associated with a decrease in natural killer T-cells and therefore be at higher risk for postoperative bacterial infection episodes [57]. Therefore, TRIM seems to depend on the degree of contamination of transfused blood with leukocytes, storage time, and cytokine content [12, 38]. However, even though the reduction of leukocyte content in blood products by prestorage leukodepletion seems to be a reasonable approach to preventing TRIM, doubts have been raised against the universal implementation of leukocyte reduction. This is because immunomodulatory effects have been described even after transfusion of leukocyte-depleted blood [58, 59]. So, it has been argued that the remaining immunomodulatory effect of blood transfusion, even after prestorage leukodepletion, could be mediated either by the few remaining leukocytes or by unidentified bioactive substances other than those present in leukocytes. Such substances could be molecules in the plasma supernatant produced or released by platelet products and might mediated immune reactions on transfusion [60]. Additionally, erythrocytes might also play a role in immunosuppression, since erythrocyte membrane phospholipids have been shown to activate macrophage-derived

phospholipids, which are potent immunoregulatory factors [4, 43, 45, 61, 62]. Moreover, allogeneic stored blood, with but also without leukodepletion, resulted in a significant TNF-α depression and IL-10 induction in an *in vitro* model of transfusion that used cultured human blood [38]. Therefore, the practice of universal leukocyte reduction has been questioned; it has not been adopted worldwide and it varies significantly among countries, taking into consideration economic factors as well [63–65]. Particularly in North America, a strong opposition against universal leukocyte reduction has been expressed by a large group of American blood bank physicians [66]. Prospective randomized studies could give some solid answers regarding the undoubtful benefit of leukodepletion, but these studies could be performed only in countries where the practice is not mandatory.

In conclusion, in the present subanalysis and taking into consideration the restrictions of the small sample size, a more liberal transfusion strategy was associated with higher IL-10 levels. Although it cannot definitely be stated that excessive transfusion therapy is responsible for the elevation of IL-10, the correlations observed show that the strength of the association between blood transfusion and IL-10 is greater than that for IL-6. Therefore, IL-10 with its potent anti-inflammatory effect may play a distinct role in the downregulation of host immunity and blood transfusion may exert its immunosuppressive effect in part by stimulating IL-10 production. However, a larger sample size and a more controlled analysis would be needed to address the question of transfusion-related immunomodulation and reproduce the findings of this preliminary report.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 473765, 10 pages
http://dx.doi.org/10.1155/2014/473765

Research Article

Modulation of Circulating Cytokine-Chemokine Profile in Patients Affected by Chronic Venous Insufficiency Undergoing Surgical Hemodynamic Correction

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Received 23 January 2014; Revised 17 February 2014; Accepted 17 February 2014; Published 25 March 2014

Academic Editor: Jana Barlic-Dicen

The expression of proinflammatory cytokines/chemokines has been reported in *in vitro*/*ex vivo* settings of chronic venous insufficiency (CVI), but the identification of circulating mediators that might be associated with altered hemodynamic forces or might represent innovative biomarkers is still missing. In this study, the circulating levels of 31 cytokines/chemokines involved in inflammatory/angiogenic processes were analysed in (i) CVI patients at baseline before surgical hemody namic correction, (ii) healthy subjects, and (iii) CVI patients after surgery. In a subgroup of CVI patients, in whom the baseline levels of cytokines/chemokines were analyzed in paired blood samples obtained from varicose vein and forearm vein, EGF, PDGF, and RANTES were increased at the varicose vein site as compared to the general circulation. Moreover, while at baseline, CVI patients showed increased levels of 14 cytokines/chemokines as compared to healthy subjects, 6 months after surgery, 11 cytokines/chemokines levels were significantly reduced in the treated CVI patients as compared to the CVI patients before surgery. Of note, a patient who exhibited recurrence of the disease 6 months after surgery, showed higher levels of EGF, PDGF, and RANTES compared to nonrecurrent patients, highlighting the potential role of the EGF/PDGF/RANTES triad as sensitive biomarkers in the context of CVI.

1. Introduction

Chronic venous disease (CVD) is one of the most prevalent medical problems in the adult population of Western European countries and USA with a significant impact on afflicted patients and on the healthcare system [1]. Dysfunction of any of the normal structures of the venous system may lead to venous hypertension and to the development of chronic venous insufficiency (CVI), an advanced form of CVD presenting with a variety of signs and manifestations ranging from varicosities up to venous ulceration [2]. The available therapeutic options include conservative therapies (phlebotonic drugs, lower limbs elastic compression), ablative surgical or endovenous procedures (sclerotherapy, laser/thermal venous shrinkage) and saphenous sparing hemodynamic surgery [3]. Although a significant effort has been made on the development and evaluation of techniques that might assist in both diagnosis and treatment of CVI, the etiopathology of this condition still needs to be clarified and CVI remains a disease with a high recurrence rate that will be possibly reduced just whenever deeper comprehension of the pathophysiological mechanisms will be obtained [4, 5].

Clinical and basic science studies overall underlie that CVI results from a complex interplay of multiple factors, including the alteration of the hemodynamic forces acting on the vein wall that can be considered as a key event in CVI development [6, 7]. It is still unclear what initiates the inflammatory process in the vein wall, but it has been shown that the persistent venous hypertension characterizing CVI leads to an inflammatory response primarily mediated by leukocytes and involving cascades of cytokines/chemokines, matrix metalloproteinases activation, and alteration of endothelial cellular functions [8–11]. Expression of pro-inflammatory cytokines and chemokines has been reported in patients with varicose veins confirming the role of inflammation in CVI [12, 13]. In a recent work, we have shown a correlation between the PDGF-BB released by patient-derived vein endothelial cell (VEC) cultures and relevant hemodynamic parameters measured *in vivo* into the venous segments from which the VEC was isolated upon surgical ablation [14].

On these bases, to elucidate the link between systemic inflammation and altered hemodynamic forces, the primary aim of this study was to evaluate the effect of a saphenous sparing surgical correction (CHIVA strategy) [15] on the levels of circulating factors related to inflammation and angiogenesis characterizing CVI in order to identify a panel of biological markers able to correlate with the disease that might complement the standard procedures for diagnosis and posttreatment follow-up of CVI patients.

2. Materials and Methods

2.1. Patients and Samples Collection. The subjects involved in this study consisted of 32 patients (for a total of 60 plasma samples) affected by primary CVI with superficial venous reflux (C2-4EpAsPr following the CEAP classification) enrolled by the Vascular Disease Center at the University of Ferrara, in accordance with the Declaration of Helsinki and with approval obtained from the University-Hospital of Ferrara. All patients underwent a complete clinical assessment to evaluate the clinical, etiology, anatomy, and pathophysiology clinical class (CEAP) and to attribute a venous clinical severity score (VCSS) [7, 16]. At the same time, patients underwent echo-color-Doppler (ECD) scanning. The patient was examined in standing position with complete scanning of the great saphenous vein (GSV) and short saphenous vein (SSV) system, including junctions and tributaries. In addition, the main trunk of the deep venous system (external iliac, common, and superficial femoral veins, popliteal and gastrocnemius veins) and the perforators were completely examined. Calf muscular pump was elicited by manual squeezing, considering reflux the detection of a reverse flow longer than 0.5 sec in whatever of the above reported superficial and deep veins [17]. At the junction, level competence of the valve was also tested by the means of Valsalva manoeuvre as previously described [18]. Limitedly on the GSV, at 15 cm from the junction with the femoral vein, we also recorded, in addition to reflux time (RT), the following hemodynamic parameters: peak systolic velocity (PSV) and end diastolic velocity (EDV) that allowed the calculation of the resistance index (RI), as previously described [14]. After clinical assessment, patients underwent Ambulatory Conservative Hemodynamic Management of Varicose Veins (CHIVA) surgery, a hemodynamic minimally-invasive approach aiming to restore the venous drainage without eliminating the saphenous system [19, 20]. Blood samples were collected from patients before and/or after surgical treatment in the presence of sodium citrate. Samples were immediately centrifuged for plasma isolation that was stored at −80°C in single-use aliquots. For some patients ($n = 6$), before surgical treatment, paired blood samples were harvested from both the forearm vein and the more prominent and incompetent GSV tributary. Plasma samples obtained from blood harvested from the forearm vein of healthy subjects (age range: 25–60 years; gender: 60% men) were used as controls.

2.2. Analysis of Cytokines and Chemokines in Plasma Samples. Plasma samples were frozen and thawed only once before performing the MILLIPLEX MAP Human Cytokine/Chemokine Panel (Merck Millipore, Billerica, MA), a bead-based multiplex immunoassay, which allows the simultaneous quantification of the following 29 human cytokines: IL-1α, IL-1β, IL-1 receptor antagonist (ra), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, EGF, Eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, CXCL10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, and VEGF. Moreover, a custom made MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore) was used to quantify the cytokines PDGF-AB/BB and RANTES (see Supplementary Table 1 available online at http://dx.doi.org/10.1155/2014/473765 for the list of the cytokines' abbreviations). Samples were processed in duplicate following the manufacturer's recommended protocols and read on a MAGPIX instrument equipped with the MILLIPLEX-Analyst Software using a five-parameter nonlinear regression formula to compute sample concentrations from the standard curves.

2.3. Network Analysis. Mapping of cytokines/chemokines into specific biological networks has been performed by using MetaCore analytical suite version 5.3 (GeneGo, MI, USA), an integrated analytical suite of algorithms based on a database of human protein-protein interactions, transcriptional factors, cell signaling, metabolism, and bioactive molecules linked to functional processes and diseases (http://www.genego.com/). Networks of cytokines/chemokines were created by using two different algorithms: (i) the network algorithm analysis generating subnetworks ranked by a P value and interpreted in terms of gene ontology processes to deduce top scoring processes that are regulated by differentially expressed proteins and (ii) the shortest paths algorithm to map the shortest path for interaction. The resulting networks were evaluated to determine which algorithm succeeded in creating modules that have higher than random saturation with the protein of interest. Networks were then graphically visualized as

Table 1: Baseline demographic/clinical characteristics of the CVI patients ($n = 32$).

	%	Mean ± SD	Range
Gender	—		
Men/women	31/69	—	—
Patient age (years)			
Men	—	50.3 ± 12.5	37/61
Women	—	58.6 ± 12.5	38/76
CEAP classification			
C2	37	—	—
C3	44	—	—
C4	19	—	—
Primary etiology	100	—	—
Superficial venous reflux	100	—	—
Reflux only	100	—	—
Hemodynamic parameters			
Peak systolic velocity (cm/sec)	—	40.63 ± 14.05	15/68
End diastolic velocity (cm/sec)	—	−17.13 ± 7.95	−35/−4
Resistance index	—	1.43 ± 0.14	1.19/1.74
Reflux time (sec)	—	2.97 ± 0.73	1.70/4.60
Clinical score (VCSS)			
5	6	—	—
6	28	—	—
7	38	—	—
8	28	—	—

nodes (proteins) and edges (connection between proteins) alongside the pattern of expression.

2.4. Statistical Analysis. Data were calculated as median, mean ± standard deviation (SD). Box plots were used to show the median and interquartile values for each group of data. The results were evaluated by using analysis of variance with subsequent comparisons by Student's t-test and with the Mann-Whitney rank-sum test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Study Population and Clinical Assessment. The main baseline demographic/clinical characteristics of the CVI patients are reported in Table 1. Overall, the 69% of enrolled patients were female with age ranging between 38 and 76 years and a mean age of 58.6 years, while male patients (31%) were characterized by age ranging between 37 and 61 years with a mean age of 50.3 years. At the baseline, assessment of VCSS ranged from 5 to 8 and corresponded to a CEAP clinical class ranging from C2 to C4. As far as the venous segments affected by CVI in our population, we found in all 32 cases the insufficiency of the GSV system, with reflux pattern involving also the SSV in 5 cases, the common femoral vein in 13 cases, the popliteal vein in 2 cases, and finally the gastrocnemius vein in 2 cases. Moreover, in our survey, the registered hemodynamic parameters at 15 cm from the sapheno-femoral junction were the following: peak systolic velocity (PSV) mean ± SD: 40.63 ± 14.05 cm/sec; end diastolic velocity (EDV), mean ± SD: −17.13 ± 7.95 cm/sec; resistance index (RI), mean ± SD: 1.43 ± 0.14; and reflux time (RT), mean ± SD: 2.97 ± 0.73 sec (Table 1).

3.2. The Levels of Selected Cytokines/Chemokines (RANTES, EGF, and PDGF) Are Higher in Varicose Vein Blood with respect to the Systemic Circulation. We have recently demonstrated that several cytokines/chemokines are significantly elevated in the plasma of patients affected by CVI with respect to healthy subjects, which suggested the presence of a systemic inflammation status in CVI patients [21]. In order to ascertain the contribution of local factors to the production and release of cytokines/chemokines found elevated in the general circulation, in a preliminary experiment, we have analyzed the levels of 31 cytokines/chemokines in paired blood samples collected both from the forearm vein and the varicose vein in a small number of patients ($n = 6$) (Figures 1(a)-1(b)). This pilot group included patients with a clinical score of 7 or 8 and C2 or C3 CEAP. Among the 31 cytokines/chemokines analyzed by multiplex assay, the following 18 were detectable, at different levels, in the plasma samples collected both from the arm and from the varicose vein in the leg: MIP-1α, IL-7, IL-8, IFN-γ, IL-12(p70), TNF-α, GM-CSF, IFN-α2, G-CSF, IL-1RA, MIP-1β, VEGF, EGF, EOTAXIN, MCP-1, CXCL10, PDGF AB/BB, and RANTES. The comparison between the levels measured in paired samples belonging to the same patient revealed the identity (with differences below 15%) between the systemic and varicose vein blood samples for most (15 out of 18) cytokines/chemokines, some of which are exemplified in

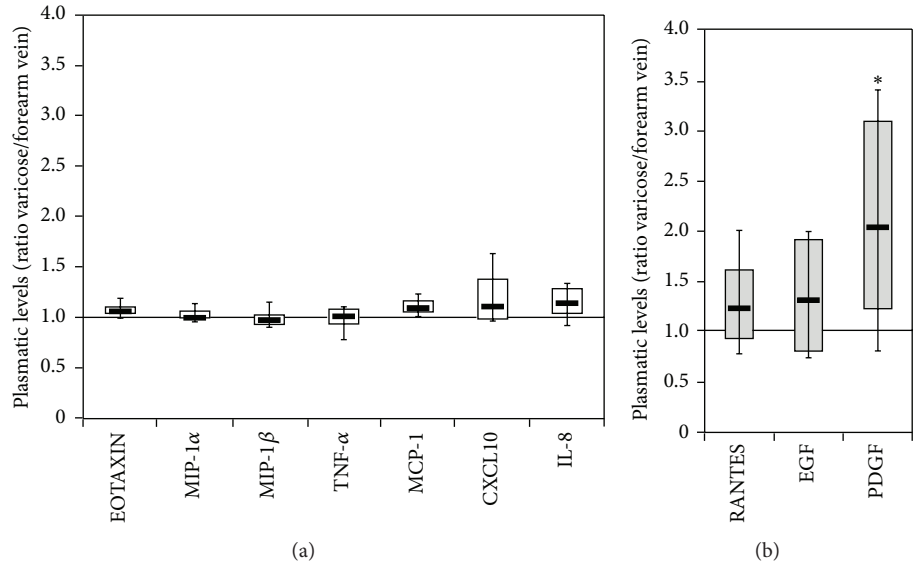


Figure 1: *Comparison between the levels of cytokines/chemokines measured in CVI patients in the varicose vein blood and in the systemic circulation*. The circulating levels of 31 cytokines/chemokines were measured in paired blood samples collected from the forearm and the varicose vein of CVI patients ($n = 6$). Data are expressed as ratio between local (varicose) and systemic (forearm) values. In (a), 7 representative cytokines/chemokines displaying the same levels at the varicose vein site and at systemic level are shown. In (b), the 3 cytokines displaying higher circulating levels ($^*P < 0.05$) at the varicose vein site with respect to the systemic level are shown. Box plots are used to show the median and interquartile values for each group of data.

Figure 1(a). For 3 cytokines/chemokines, we observed a mean increment between 20 and 35% for RANTES and EGF and a mean increment of 108 ± 24% for PDGF AB/BB (Figure 1(b)). This preliminary analysis demonstrates that the systemic profile matched the local profile for the great majority of cytokines and suggests that the increase of PDGF, RANTES, and EGF either contributes to the pathogenesis of the CVI or represents the response to the local vein damage.

3.3. Downmodulation of the Circulating Levels of Most Cytokines/Chemokines after Surgical Hemodynamic Correction. All CVI patients enrolled in the study underwent CHIVA surgery, a conservative and minimally invasive therapeutic option for CVI aiming to restore the physiological lower limbs venous drainage without eliminating the saphenous system. Briefly, the functional restoration of the GSV was obtained once the incompetent GSV tributaries were flush ligated and/or once the reflux, which is coming from an incompetent saphenofemoral confluence, was suppressed by high ligation. In paired plasma samples harvested from forearm vein of CVI patients ($n = 13$) before and after (mean of 33 days) CHIVA, unexpectedly we documented a further significant increase (≥2 folds) with respect to presurgical levels for 5 cytokines: IL-8 (mean ± SD increase: 2 ± 1.2), PDGF (mean ± SD increase: 3 ± 3.2), EGF (mean ± SD increase: 3 ± 2.6), RANTES (mean ± SD increase: 7 ± 7.2), and VEGF (mean ± SD increase: 4.4 ± 7.5) (Figure 2). Since these cytokines are known to play key roles in endothelial cell biology (in particular in promoting angiogenesis and endothelial cell remodeling), it is possible to suppose that their increase is a response to the surgical procedure. It is also noteworthy that 3 (EGF, PDGF, RANTES) out of 5 of these cytokines were those found elevated in the varicose vein as compared to the forearm vein plasma (Figure 1(b)).

However, the situation changed drastically when the cytokine and chemokine profile was analyzed in a group of patients ($n = 9$) 6 months after CHIVA and compared with the levels measured in healthy individuals and in pre-CHIVA patients (Figure 3). It should be underlined that 6 months after CHIVA, the clinical evaluation and the vascular parameters of the patients resulted significantly improved as reveled by (i) clinical score of 0 or 1, (ii) C0 or C1 CEAP clinical class, and (iii) hemodynamic parameters (RT, mean ± SD: 1.39 ± 0.53 sec; PSV, mean ± SD: 16.81 ± 6.51 cm/sec; EDV, mean ± SD: 8.31 ± 3.27 cm/sec) with the exception of one patient reporting a clinical score of 3 and C3 CEAP, with recurrent disease for failure of the surgical treatment. In line with the clinical observations, we found that the levels of 11 cytokines and chemokines were significantly ($P < 0.05$) lower with respect to the group of pre-surgical CVI patients, and for some cytokines/chemokines (including EGF, PDGF, and RANTES), the levels were not significantly different from the levels found in healthy controls (Figure 3(a)). Only three cytokines (G-CSF, CXCL10, and MCP-1) persisted at levels comparable to those measured in patients before CHIVA (Figure 3(b)). These data indicate that CHIVA is able to correct either completely or partially the circulating levels of several cytokines/chemokines altered in CVI. A network analysis of the cytokine pathways revealed the key role of RANTES as hub of the system (Figure 4(a)), with a strong direct interaction between PDGF and EGF (Figure 4(b)).

In considering the potential link between cytokines/chemokines modulation and the clinical course after CHIVA,

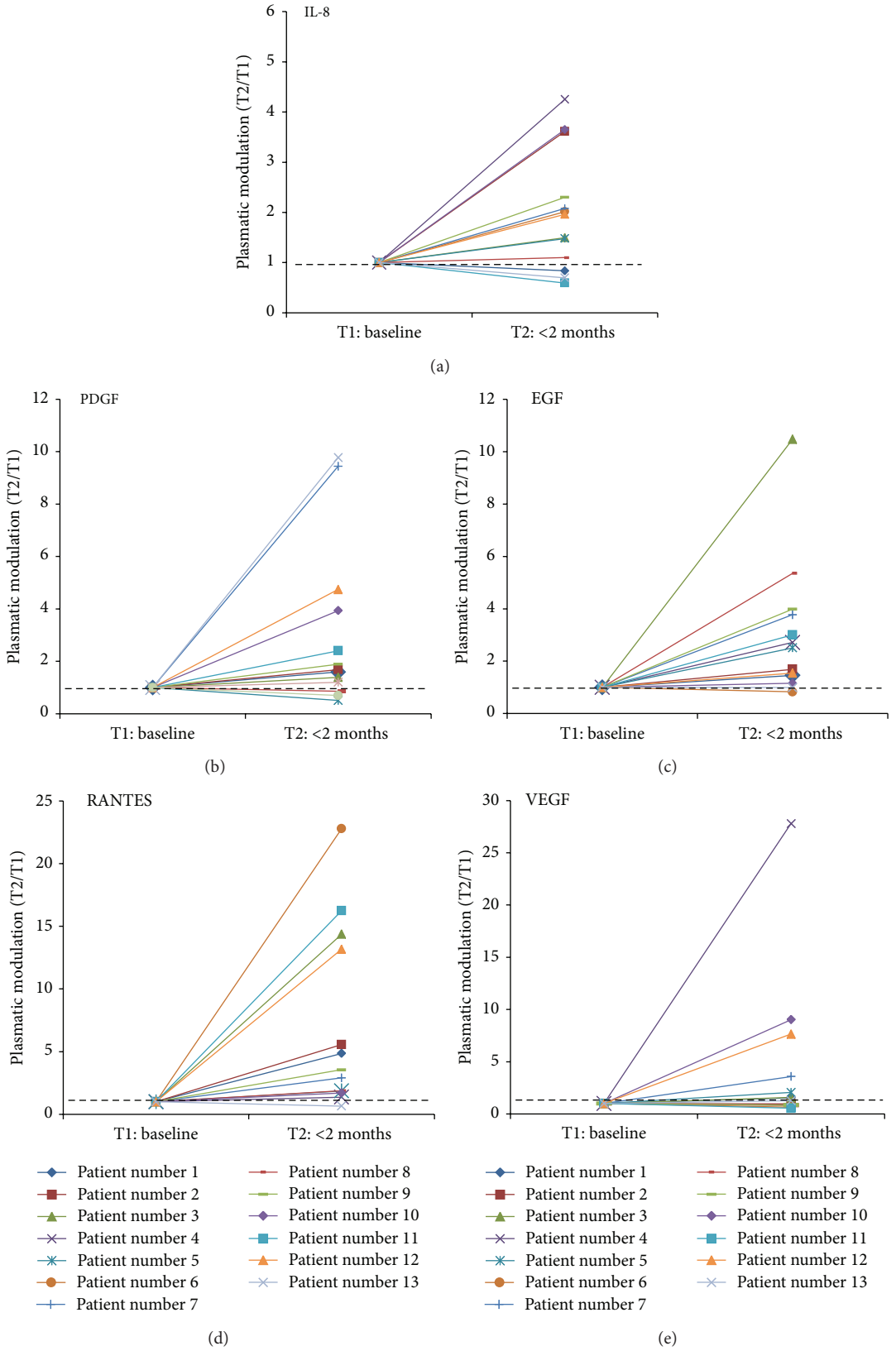
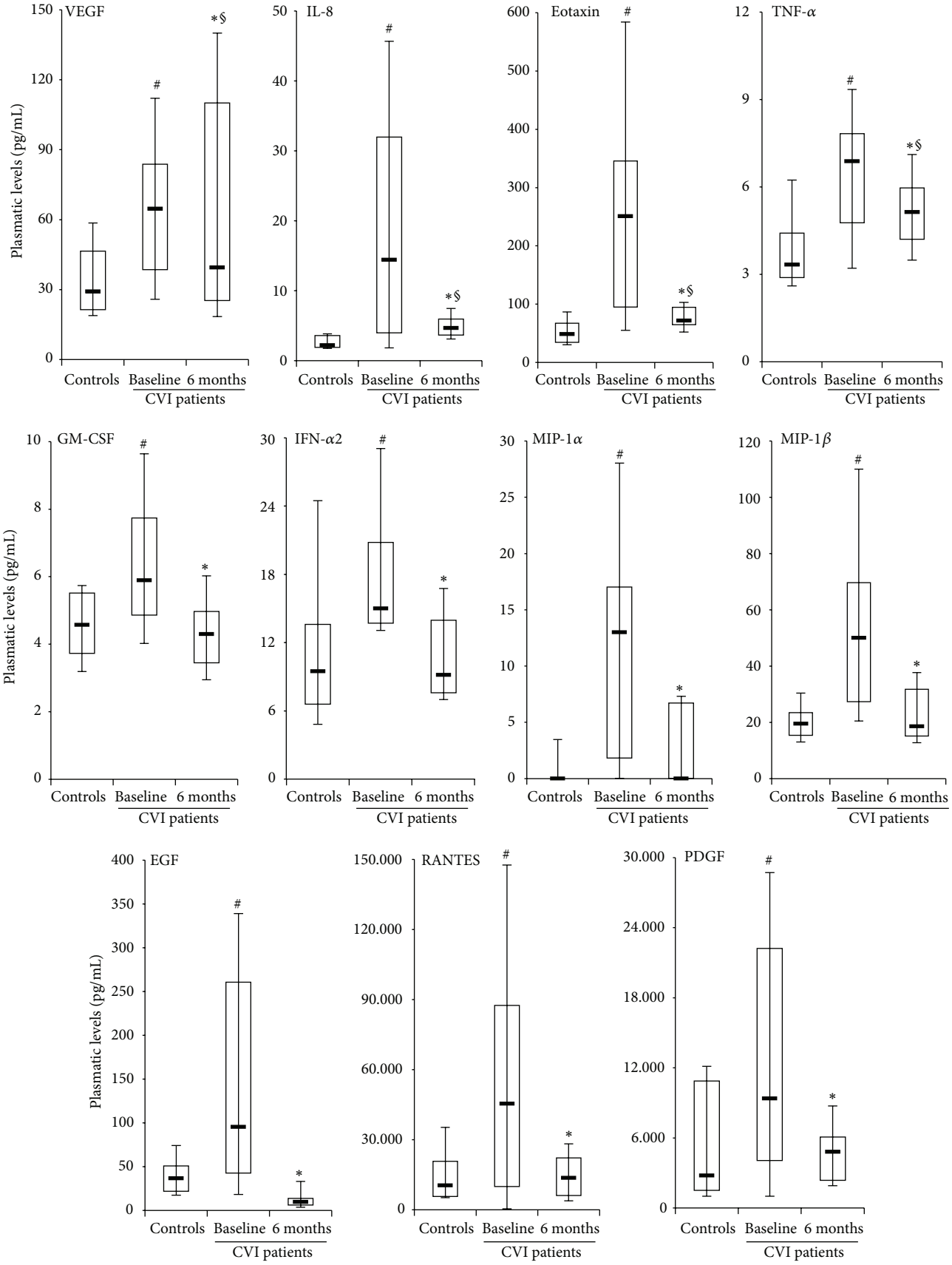


FIGURE 2: *Modulation of circulating cytokines/chemokines in CVI patients after <2 months of surgical hemodynamic correction.* The circulating levels of cytokines/chemokines were monitored in paired plasma samples of CVI patients ($n = 13$) before (at baseline, T1) and after (<2 months, T2) CHIVA. Data are shown as plasmatic modulation (T2/T1) for each patient. Hatched line indicates the baseline level set to 1.



(a)

Figure 3: Continued.

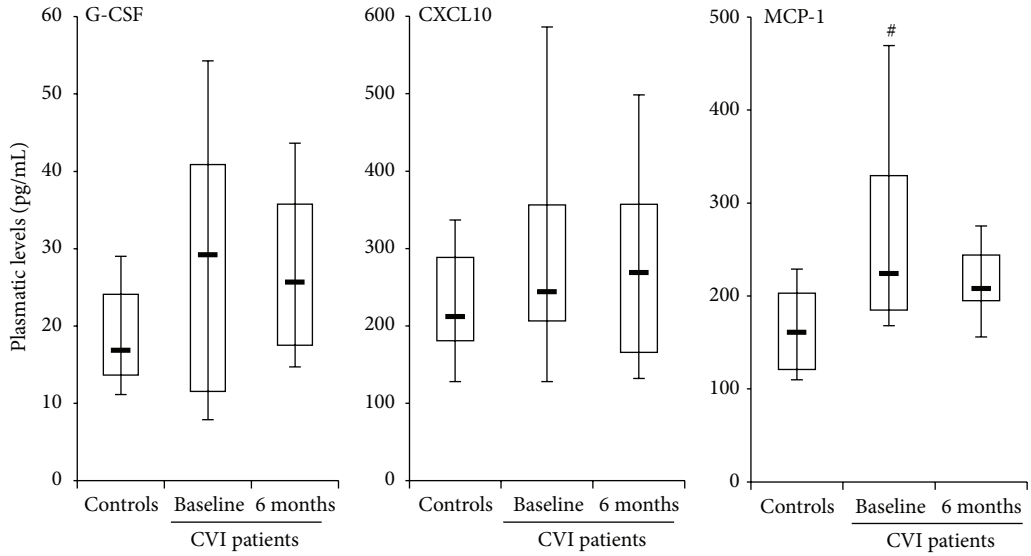


Figure 3: *Evaluation of circulating cytokines/chemokines in CVI patients 6 months after surgical hemodynamic correction.* The circulating levels of cytokines/chemokines were assessed in plasma samples harvested from forearm vein of healthy subjects (controls) and of CVI patients before (baseline) and after (6 months) CHIVA. In (a), the 11 cytokines/chemokines displaying lower levels after CHIVA, compared to baseline levels, are shown; $^{*}P < 0.05$ compared to baseline; $^{\$}P < 0.05$ compared to controls. In (b), the 3 cytokines/chemokines displaying levels after CHIVA not significantly different compared to baseline levels are shown. In (a) and (b), horizontal bars are median, upper, and lower edges of box that are 75th and 25th percentiles, lines extending from box are 10th and 90th percentiles; $^{\#}P < 0.05$ compared to the controls.

it is noteworthy that the only patient who exhibited a rapid clinical recurrence at 6 months of follow-up, as indicated by the clinical score 3, presented significantly higher levels (fold of increase >2) of EGF, PDGF, and RANTES with respect to the mean values measured in the patients with the best clinical parameters (clinical score 0-1) (Figure 5).

4. Discussion

The links between inflammation, endothelial activation/damage, and mechanical stretch/pressure in the context of CVI still need to be elucidated not only in terms of molecular mediators involved but also in terms of progression of the events. Incompetent valves with saphenous reflux (considered one of the most important hemodynamic factors in CVI) lead to endothelial activation and inflammation [2, 6–11], but there might be preexistent (inflammatory) conditions that might render the endothelium weaker and therefore more prone to lead to valve dysfunction. The effect of disturbed flow on vascular endothelium has been deeply evaluated *in vitro* in models of standard endothelium [11, 22–24] and, more recently, in an *ex vivo* model of primary venous endothelial cells (VEC) isolated from surgical saphenous specimens of CVI patients [12, 14]. Moreover, higher expression of proinflammatory cytokines/chemokines has been demonstrated at RNA level in varicose veins compared to control veins [13]. In further studies performed in the pathologic VEC model, we suggested the link between hemodynamic forces and the proinflammatory status of CVI, documented by a correlation between key hemodynamic parameters and the *ex vivo* endothelial release of cytokines/chemokines, underlining in particular the possible contribution of soluble mediators such as PDGF-BB and osteoprotegerin [14, 25], a key regulator of the two TNF family members RANKL and TRAIL [26, 27]. Overall, these *in vitro/ex vivo* evidences support the hypothesis of the presence of circulating factors involved in the inflammatory/angiogenic processes related to CVI that are released (also, but perhaps not only) by the pathological endothelium that might be able to characterize the disease. In this light, we here demonstrate that, within the plasmatic cytokines/chemokines upregulated in CVI patients compared to healthy controls, the circulating levels of selected factors (including IL-8, PDGF, EGF, VEGF, and RANTES) were further increased in a short time follow-up (<2 months) after hemodynamic correction by CHIVA, perhaps as direct consequence of the surgical procedure since they are all factors involved in endothelial remodeling. On the contrary, in patients assessed 6 months after CHIVA, with the exception of MCP-1, G-CSF, and CXCL10, we documented a significant decrease of the cytokines/chemokines, returning, in some cases, to levels similar to healthy individuals. These observations prove that the hemodynamic correction is able to restore the normal levels of several inflammatory/angiogenic factors characterizing CVI. Of interest, in a patient that at the 6 months after CHIVA presented a relapse of the disease (clinical score 3), we observed higher levels of EGF, PDGF,

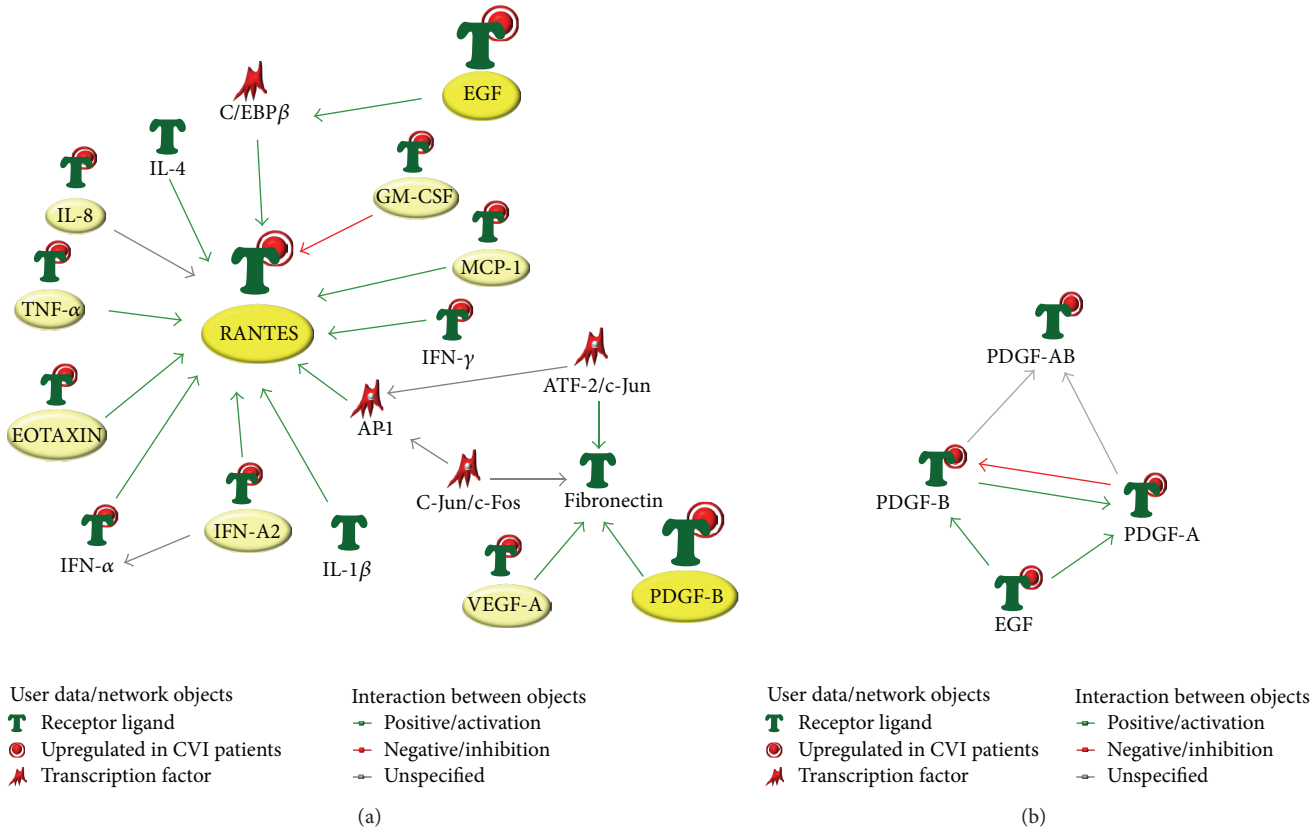


FIGURE 4: *Network analysis of the cytokines/chemokines differentially expressed in the context of CVI.* The identified panel of cytokines/chemokines characterizing CVI was assessed for network analysis. In (a), the top-score biological network generated by using the analysis network algorithm illustrating the connections among the differentially expressed cytokine/chemokine highlighting the role of RANTES as hub. In (b), proteins network between PDGF and EGF generated by the shortest path algorithm is shown.

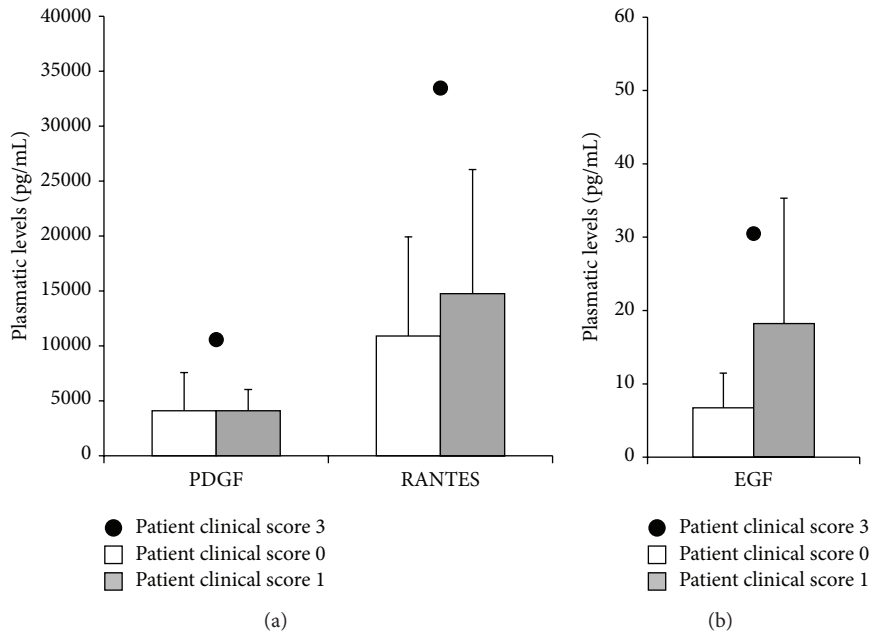


FIGURE 5: *Highest levels of PDGF, RANTES, and EGF in one CVI patient showing recurrence of the disease 6 months after CHIVA.* The plasmatic levels of PDGF, RANTES, and EGF assessed in the CVI-recurrent patient (clinical score 3) are shown in comparison to the means ± SD levels assessed in patients reporting clinical score 0 or 1.

and RANTES, with respect to the patients after CHIVA characterized by clinical score 0-1. It is important to underline that the findings reported in the present study have been obtained comparing healthy subjects and groups of CVI patients monitored at the same season (winter) since in preliminary analysis, we observed a "season-dependent" effect on the circulating levels of the considered cytokines/chemokines, in agreement with previous studies [28–30].

We are aware about the limitations of this pilot study, mainly due to the low number of subjects and the short times of the postsurgery follow-up of CVI patients due to the seasonal limitation for a proper comparison of the cytokines/chemokines levels. However, our results suggest a significant link between *in vivo* hemodynamic forces and increased circulating levels of inflammatory/angiogenic mediators (including EGF, PDGF, and RANTES). Although these circulating factors likely represent the response to CVI inflammation, we cannot exclude their contribution to the pathogenesis of varicose veins. Nonetheless, we here propose the potential role of a panel of selected cytokines, including EGF, PDGF, and RANTES, as biomarkers in the characterization of the disease progression and in the postsurgery monitoring of patients to predict disease recurrence (once their levels increase). This hypothesis is furthermore supported by the evidence that, even though we have demonstrated that the systemic profile matched the local profile, these three molecules showed the major levels at the varicose vein site. Of note, since RANTES acts as chemotactic molecule for leukocytes [13, 31], it actually appears to represent one of the hubs in the cytokine network (Figure 4(a)) and a link between the endothelial disease and the inflammatory process.

These preliminary observations are therefore the proof of concept for future clinical studies involving a larger cohort of patients with the aim of identifying CVI-specific circulating biomarkers to be used in the clinical practice at the diagnostic level and during the post-treatment follow-up of CVI patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 181450, 11 pages
http://dx.doi.org/10.1155/2014/181450

Review Article

Roles of the Chemokine System in Development of Obesity, Insulin Resistance, and Cardiovascular Disease

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Received 14 December 2013; Accepted 4 February 2014; Published 16 March 2014

Academic Editor: Mizuko Mamura

The escalating epidemic of obesity has increased the incidence of obesity-induced complications to historically high levels. Adipose tissue is a dynamic energy depot, which stores energy and mobilizes it during nutrient deficiency. Excess nutrient intake resulting in adipose tissue expansion triggers lipid release and aberrant adipokine, cytokine and chemokine production, and signaling that ultimately lead to adipose tissue inflammation, a hallmark of obesity. This low-grade chronic inflammation is thought to link obesity to insulin resistance and the associated comorbidities of metabolic syndrome such as dyslipidemia and hypertension, which increase risk of type 2 diabetes and cardiovascular disease. In this review, we focus on and discuss members of the chemokine system for which there is clear evidence of participation in the development of obesity and obesity-induced pathologies.

1. Introduction

More than 35% of adults and almost 17% of youth in the United States were reported to be obese in 2012 (http://www.cdc.gov/obesity/data). Obesity is an independent risk factor for metabolic disorders including insulin resistance, type 2 diabetes, hypertension, adverse plasma lipid concentrations, and cardiovascular disease (Figure 1) [1]. Given the high prevalence of obesity and its health risks, it is important to understand molecules, signals, and mechanisms leading to impaired and/or aberrant adipose tissue functions that support excessive body weight gain.

Chemokines are small-secreted molecules that mediate cell recruitment by interacting with their cognate chemokine receptors. They were originally described as mediators of leukocyte migration during the immune response. Subsequently, chemokines were discovered to control movement of other cell types in many different contexts. The human chemokine system comprises about 50 chemokines and 20 receptors belonging to the seven-transmembrane G protein-coupled receptor family. Based on the motif patterns involving two amino-terminal cysteine residues, chemokines are classified into the following four subfamilies: CXC, CC, C, and CX3C, where X is any amino acid residue. Chemokines appear to exhibit a high degree of functional redundancy. Some chemokines have a one-to-one specificity (specific receptor), while for others multiple chemokine ligands binding to the same receptor (shared receptor) have been reported. When multiple ligands interact with a single receptor, different responses are produced because ligands have different binding affinities for the receptor [2].

In this review we highlight the chemokine-chemokine receptor axes that facilitate development of obesity by supporting chronic low-grade adipose tissue inflammation and those that are a link and/or a key contributing factor to insulin resistance, type 2 diabetes, and cardiovascular disease.

2. The Chemokine System in Development of Obesity

The root cause of obesity is a prolonged imbalance between caloric intake and energy expenditure which results in increased lipid storage and adipose tissue expansion. Adipose tissue is composed of two main cell types, adipocytes and stromovascular mononuclear cells (i.e., resident leukocytes). Adipose tissue occurs in white and brown forms that serve different functions [3].

The main role of brown adipose tissue (BAT) is adaptive thermogenesis, the process of regulated heat production in

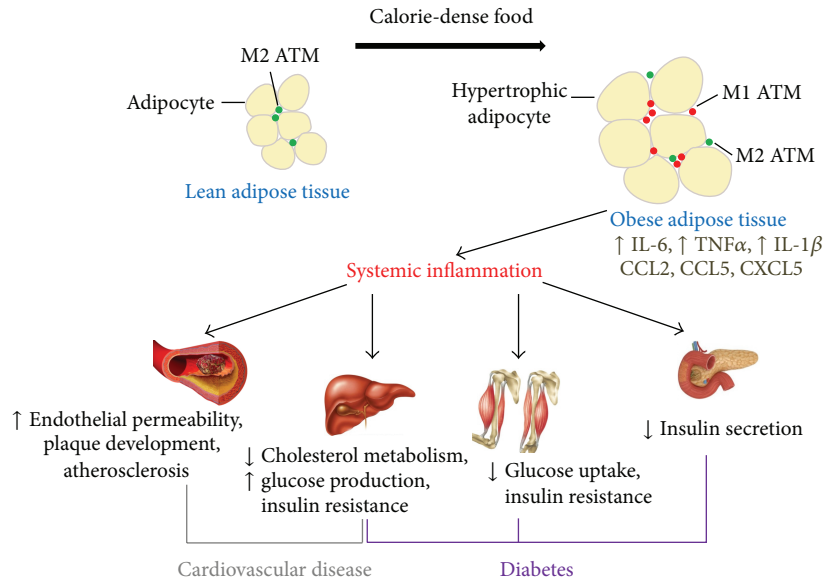

Figure 1: *Obesity contributes to development of diabetes and cardiovascular disease.* Adipose tissue is composed of two main cell types, adipocytes and stromovascular mononuclear cells (i.e., resident leukocytes). Adipose tissue macrophages (ATMs) are the most frequent leukocyte subtype in fat tissues. Normal adipose tissue is populated with the alternatively activated M2 ATMs. Persistent or frequent consumption of calorie-dense food results in obesity that is associated with increased adiposity which includes adipose tissue hypertrophy and influx of proinflammatory monocytes that mature to classically activated M1 ATMs. Obesity induces production of proinflammatory cytokines (i.e., IL-6, TNFα, and IL-1β) and several chemokines including CCL2, CCL5, and CXCL5 among others by adipocytes and immune cells trigger adipose tissue inflammation, which when prolonged progresses to systemic inflammation that affects (i) vasculature increasing permeability of endothelium, thereby triggering plaque development and cardiovascular disease; (ii) anabolic actions of insulin and insulin signaling in metabolic tissues including liver and skeletal muscle, causing insulin resistance that manifests as impaired glucose disposal in muscle and altered cholesterol and glucose metabolism in the liver, which in turn triggers hyperinsulinemia, hyperglycemia, and hyperlipidemia that all contribute to type 2 diabetes and cardiovascular disease; and (iii) pancreas, decreasing insulin secretion that leads to hyperglycemia, which is a hallmark of diabetes.

response to the environmental temperature or diet. There are three subcategories of adaptive thermogenesis. Cold exposure induces shivering thermogenesis in skeletal muscle and nonshivering thermogenesis in brown adipocytes. Overfeeding triggers diet-induced or metabolic thermogenesis, which allows excess energy received in the form of food to dissipate as heat and thereby this form of thermogenesis protects from obesity [4].

White adipose tissue (WAT) provides an important energy depot in the form of stored lipids [3]. In addition to energy storage, WAT serves as an endocrine organ that produces adipokines, signaling molecules secreted by adipocytes, which have multiple effects at both local and systemic levels. Excessive fat uptake results in adipocyte expansion (hypertrophy) and in overproduction and secretion of signals that recruit inflammatory cells into WAT, triggering low-grade chronic inflammation that is mediated by the cells of innate and adaptive immune systems [5, 6].

In normal adipose tissue, the main resident immune cell subtypes are the alternatively activated $CD163^+$ and/or $CD206^+$ (M2) adipose tissue macrophages (ATMs), regulatory T cells (Treg), and T helper 2 (Th2) cells. These resident leukocytes produce anti-inflammatory interleukin-10 (IL-10) and transforming growth factor β (TGFβ) that maintain adipose tissue homeostasis. Aberrant adipose tissue expansion and hypoxia associated with the excessive adipose tissue growth as well as activation of oxidative stress responses orchestrate the production of proinflammatory cytokines like interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα) as well as chemokines. These signals support influx of proinflammatory monocytes, which in WAT mature to classically activated $CD40^+$ and/or $CD11c^+$ (M1) ATMs, as well as recruitment of neutrophils, mast cells, natural killer cells, and dendritic cells. In addition to innate immune cells, adaptive response immune cells including CD8 effector memory, T helper 1 (Th1), and B cells are all increased in the adipose tissue during the course of obesity. Thus, overfeeding triggers qualitative and quantitative alterations in the adipose tissue stromovascular fraction that, together with adipocyte hypertrophy, fuel chronic low-grade adipose tissue inflammation that exacerbates obesity [6].

Chemokines including CCL2, CCL5, CCL7, CCL8, CCL11, CCL13, CXCL5, CXCL8, and CXCL10 are upregulated in different depots of adipose tissue. Serum levels of these chemokines are dramatically increased in obese versus lean individuals. Expression of chemokine receptors CCR1, CCR2, CCR3, and CCR5 is elevated on inflammatory cells in omental and subcutaneous adipose tissues of obese patients [7, 8].

There are several mouse models that are used to study development of obesity and obesity-induced comorbidities. The model that most closely resembles human obesity and obesity-linked pathologies including metabolic syndrome is the inbred C57BL/6 strain fed the obesogenic high-fat diet containing 60% fat. While this model is useful for studies addressing development of obesity in humans, there are also differences: (i) mice are typically individually housed and in relatively small cages, which restricts social interaction and physical activity; (ii) the normal housing temperature is 20–23°C, which is several degrees lower than their thermoneutral temperature (29–32°C), and animals consequently expend energy to keep warm; (iii) food is readily available at all times, which is not always true for humans; and (iv) eating habits and psychological factors that control food intake and overeating in mice and in man may not be the same [9].

Using the mouse model of diet-induced obesity, it was demonstrated that targeted deletion of the chemokine *Ccl2* [10, 11] or its receptor *Ccr2* [12] decreased inflammatory ATM content and adipose tissue inflammation. Conversely, adipose tissue-specific overexpression of CCL2 increased inflammatory ATM content in adipose tissues [13]. Thus, the CCL2-CCR2 axis was suggested to be the main promoter of inflammatory cell recruitment into fat tissue in obese mice. However, recent studies have produced opposite results. Genetic inactivation of CCL2 was demonstrated to not interfere with obesity-associated monocyte influx into WAT [14]. Furthermore, $Ccr2^{-/-}$ mice fed an obesogenic high-fat diet had fewer macrophages in WAT relative to control wild-type mice; however, lack of CCR2 did not decrease ATM content to the levels detected in lean mice [14, 15]. This indicates that proinflammatory monocyte influx into hypertrophic adipose tissue is, in addition to the CCL2-CCR2 axis, also regulated by other chemokine-chemokine receptor interactions.

Indeed, the chemokine receptor $Cxcr2^{-/-}$ bone marrow chimeras show decreased obesity-induced adipose tissue inflammation [16]. Furthermore, the chemokine receptor CCR5-mediated signaling in the adipose tissue is thought to maintain obesity-induced inflammation. As in obese individuals [17], expression of CCR5 and its ligands is greatly increased in the WAT of high-fat diet-fed obese mice. Furthermore, high-fat diet feeding causes a robust increase in $CCR5^+$ ATMs in hypertrophic WAT. Moreover, lack of CCR5 expression in myeloid leukocytes alone is associated with a marked reduction in proinflammatory monocyte infiltration into WAT and protects mice from the obesity-induced adipose tissue inflammation [18]. These data suggest that $CCR5^+$ ATMs contribute to development of obesity and obesity-induced adipose tissue inflammation. However, a study by Kennedy et al. shows that CCR5 has a minor role in regulating M1 ATMs infiltration but increases influx of $CD4^+$ T into hypertrophic adipose tissue [19], indicating that targeting CCR5 only may not be the best approach for treating obesity-induced adipose tissue inflammation.

3. The Chemokine System in Insulin Resistance

Insulin, a hormone produced by β-cells in the pancreas, is central in regulation of carbohydrate and fat metabolism. Insulin regulates uptake of blood glucose by metabolic tissues including liver, skeletal muscles, and fat tissues. Obesity compromises metabolic homeostasis by altering glucose absorption and insulin sensitivity in these tissues.

The obesity-induced macrophage phenotype switch in expanding adipose tissue involves a decrease in anti-inflammatory M2 ATM content paralleled with an increase in proinflammatory M1 ATM content. Subsequent recruitment of other innate and adaptive immune cells together with the macrophage phenotype switch triggers systemic inflammation, which decreases insulin sensitivity in adipose as well as in other insulin-sensitive metabolic tissues. Prolonged and/or long-term nutrient excess exacerbates adipose tissue inflammation which interferes with anabolic actions of insulin and insulin signaling in metabolic tissues, causing insulin resistance that manifests as impaired glucose disposal in muscle and enhanced triglyceride lipolysis in adipose tissue. Hyperinsulinemia, hyperglycemia, and hyperlipidemia, which often are a result of insulin resistance, all contribute to metabolic syndromes including development of type 2 diabetes and cardiovascular disease, both of which are global health problems (Figure 1) [20].

Because insulin resistance has a pivotal role in the pathogenesis of type 2 diabetes, efforts are being made to elucidate factors responsible for obesity-induced insulin resistance. Several lines of evidence suggest that the chemokine system links obesity to insulin resistance by regulating macrophage functional responses as well as by controlling proinflammatory monocyte influx and, with that, M1 ATM content in adipose tissues [21].

Genetic inactivation of CCL2-CCR2 signaling [11, 12, 22] or inhibition of this axis [23, 24] by pharmacological approaches was shown to improve insulin sensitivity in obese mice, whereas overexpression of CCL2 impaired glucose metabolism in this mouse model [13]. However, recent data suggest that lack of CCL2 does not improve metabolic function [15]. Furthermore, CCR2 deficiency was not shown to eliminate insulin resistance. Together this shows that glucose metabolism is regulated by CCL2-CCR2 independent signals as well [25].

Deficiency in CCR5 was also shown to improve obesity-induced insulin resistance in mice. $Ccr5^{-/-}$ mice and $Ccr5^{-/-}$ bone marrow chimeras fed the obesogenic diet both showed resistance to obesity-induced insulin resistance and type 2 diabetes [18]. Moreover, myeloid leukocyte CCR5 deficiency alleviated high-fat diet-induced insulin resistance and hepatic steatosis [18]. In contrast to this study, Kennedy et al. recently reported that obese $Ccr5^{-/-}$ mice develop systemic glucose intolerance [19]. Thus, inhibition of CCR5 signaling can be considered as a novel intervention to treat insulin resistance and type 2 diabetes only after roles of CCR5 in human adipose tissue are fully identified.

Studies have shown that CXC motif chemokine ligand 5 (CXCL5) and its cognate chemokine receptor CXCR2 also support development of insulin resistance. CXCL5 serum levels are markedly increased in obese compared to lean subjects [26]. Treatment of obese, insulin-resistant mice with either neutralizing anti-CXCL5 antibodies or CXCR2 antagonist decreased fasting glucose levels and improved insulin sensitivity. *Cxcr2*$^{-/-}$ mice were shown to be protected from diet-induced insulin resistance and diabetes [16, 26]. Thus, this finding suggests that inhibition and/or neutralization of CXCL5 may be considered as a therapeutic tool for treatment of metabolic syndrome.

The expression of the CXC motif chemokine ligand 14 (CXCL14) was shown to be elevated in the high-fat diet-fed WAT of obese mice. *Cxcl14* inactivation improved insulin sensitivity in high-fat diet-fed female but not male mice. In addition, *Cxcl14*$^{-/-}$ mice were also protected from diet-induced hyperglycemia, hyperinsulinemia, and hypoadiponectinemia. Interestingly, overexpression of CXCL14 in skeletal muscle restored obesity-induced insulin resistance in *Cxcl14*$^{-/-}$ mice [27], suggesting that CXCL14 is important during glucose uptake in skeletal muscle and by doing so this chemokine regulates glucose metabolism.

Although the chemokine system is thought to participate in the development of insulin resistance by sustaining adipose tissue inflammation, the CX3C chemokine ligand 1 (CX3CL1) and its cognate chemokine receptor CX3CR1 were demonstrated to regulate pancreatic β-cell insulin secretory pathway in which CX3CL1 stimulates CX3CR1 to increase insulin secretion. High-fat diet failed to trigger obesity in *Cx3cr1*$^{-/-}$ mice and the CX3CL1-CX3CR1 axis did not seem to promote inflammatory macrophage accumulation in adipose tissue or liver, thereby preventing inflammation-induced insulin resistance. However, *Cx3cr1*$^{-/-}$ mice fed either regular chow (10% fat) or a high-fat diet developed glucose intolerance while exhibiting normal insulin sensitivity. This suggests that deficient insulin secretion causes hyperglycemia in *Cx3cr1*$^{-/-}$ mice. Furthermore, islets isolated from *Cx3cr1*$^{-/-}$ mice produced less insulin in response to various stimuli. *In vivo* administration of soluble CX3CL1 increased plasma insulin levels and improved glucose tolerance [28]. Thus, this finding suggests that it may be possible to both improve glucose metabolism and prevent type 2 diabetes by facilitating CX3CR1 signaling.

4. The Chemokine System in Cardiovascular Disease

4.1. Chemokines and Chemokine Receptors Promoting Plaque Development. The link between obesity and atherosclerotic cardiovascular disease extends beyond the overlapping incidence. These two conditions share similar pathophysiological pathways. Obesity and atherosclerosis used to be described as simple lipid-storage diseases involving triglycerides in adipose tissue and low-density lipoprotein (LDL), also known as "bad cholesterol," in atheroma. However, obesity and atherosclerosis are now viewed as chronic inflammatory processes supported by activation of innate and adaptive immunity [29–31].

Deposition and modifications of LDL in the subendothelium and the consequent proinflammatory reactions of cellular elements in the arterial wall promote influx of inflammatory leukocytes into the vascular subendothelium, which supports the development of multifocal atherosclerotic plaques. Most plaques are asymptomatic and some become obstructive but only a few become thrombosis-prone and cause complications including acute myocardial infarction and stroke [32, 33].

Statins are widely used to treat atherosclerosis. Large randomized clinical trials have documented their benefits in patients at risk for or presenting established atherosclerotic cardiovascular disease. Statins lower plasma cholesterol by reversibly inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis in the liver. They have also been shown in both experimental and clinical studies to have potent anti-inflammatory, vasodilatory, and antiplatelet effects that are independent of their lipid-lowering effects. These non-lipid-lowering effects depend on HMG-CoA reductase inhibition in tissues other than the liver. The effects include improvement of endothelial function, decreased vascular smooth muscle cell proliferation, attenuated vascular inflammation, increased plaque stability, and prevention of thrombus formation. Despite numerous beneficial effects, recent studies investigating effects of long-term intensive statin therapy on atherosclerosis burden have demonstrated that statins do not eliminate plaque burden completely, leaving patients prone to adverse cardiovascular events [34, 35]. Thus, efforts are being made to develop new interventions that will eliminate this risk. For this purpose, suitable preclinical models that allow identification of mechanisms, molecules, and signals supporting plaque development have been generated.

Atherosclerosis-prone apolipoprotein E-deficient (*ApoE*$^{-/-}$) and low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice have been developed and extensively used in studies of atherosclerotic plaque development and used to evaluate novel drug targets. The use of these models also showed that there are differences between mouse and human atherogenesis. Lesions in mice are not prone to spontaneous plaque disruption with thrombosis that complicates human disease. Mouse studies mostly focus on aortic plaques, whereas the most important clinical consequences of atherosclerosis in humans arise from lesions in the coronary, carotid, and/or cerebral arteries. The proximal left anterior descending coronary artery in humans which is a frequent site of lesion formation contains a considerable population of intimal smooth muscle cells, which is a major difference from mouse arteries. Despite these differences, consumption of the Western diet, dense in fat and carbohydrates, triggers hypercholesterolemia and vascular inflammation that drive plaque development in mice and humans [36, 37].

Inflammatory cells involved in atherogenesis are highly heterogeneous. In mice, a proinflammatory subset of monocytes induced by hypercholesterolemia preferentially

furnishes the precursors of lesional macrophages and foam cells, but the fates and functions of this monocyte subset and its human equivalent remain under intense investigation. Plaque macrophages also have proinflammatory functions, characteristic of M1 ATMs in inflamed obese adipose tissue. They produce high levels of proinflammatory cytokines including interleukin-1β (IL-1β) and TNFα. Some mononuclear phagocytes in plaques perform functions of the antigen-presenting dendritic cells. Other leukocyte classes including T and B lymphocytes and mast cells accumulate mostly in advanced atheroma, but these cells are less abundant than phagocytes. Lesional Th1 cells are proatherogenic and Tregs are atheroprotective. The roles of Th2 and Th17 still remain controversial. The B1 subset of B cells is thought to be atheroprotective, and the B2 subset of B cells seems to be proatherogenic [38].

Chemokine-mediated recruitment and accumulation of leukocytes in the arterial wall is an important mechanism supporting atherosclerosis progression. Several chemokines including CCL2, CCL5, and CX3CL1 as well as their cognate chemokine receptors CCR2, CCR5, and CX3CR1 are expressed in mouse and human atherosclerotic lesions [39–44]. Proatherogenic *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ strains fed the Western diet (21% fat, 0.3% cholesterol) that are deficient in CCL2 [45] or CCR2 [46, 47] show reduced atherosclerosis burden compared to wild-type controls. Single nucleotide polymorphisms (SNPs) in the promoter of CCL2, CCL2-2518G [48], and in the open reading frame of CCR2, CCR2-V64I [49], have been associated with increased risk of myocardial infarction in humans. Furthermore, antagonism or deficiency in CCR5 or CCL5 or CX3CL1 or CX3CR1 also reduces atherosclerosis in mice [50–54]. In humans, the gain-of-function polymorphism CCL5-G403A affects basal CCL5 expression levels and is associated with a higher risk of coronary artery disease (CAD) [55, 56], whereas the loss-of-function polymorphism CCR5Δ32 seems to be associated with a lower risk of myocardial infarction [56, 57]. The analysis of Framingham Heart Study Offspring Cohort indicated that two SNPs of CX3CR1, V249I and T280 M, are associated with a markedly reduced risk of CAD [58–60]. However, two other studies showed that these polymorphisms were not associated with peripheral arterial disease but were associated with increased risk of restenosis after coronary stenting [61, 62].

The main leukocyte subset initiating and driving plaque progression is monocytes. Expression of CCR2, CCR5, and/or CX3CR1 supports differential monocyte subset recruitment and functions in the plaque. Ly6C^{hi}CCR2^{+}CX3CR1^{low} monocytes (or CD14^{hi}CD16^{-} in humans) more efficiently infiltrate sites of inflammation (inflammatory monocytes), while Ly6C^{low}CCR2^{-}CX3CR1^{hi} monocytes (or CD14^{+}CD16^{+} in humans) have a major surveillance function in homeostasis (resident monocytes). Ly6C^{hi} monocytes are dramatically increased in hypercholesterolemic mice. This monocyte subset uses in addition to CCR2 also CCR5 and CX3CR1 to home to plaques. Interestingly, Ly6C^{low} monocytes can also enter plaques, although they do so less frequently than Ly6C^{hi} monocytes and seem to require CCR5 rather than CX3CR1. Although both Ly6C^{hi} and Ly6C^{low} monocyte subsets can differentiate into CD11c^{+} dendritic cells, Ly6C^{low} monocytes are more prone to becoming CD11c^{+} cells within lesions, indicating functional differences between these two monocyte populations [63, 64]. In addition, CX3CL1 is a unique chemokine that exists in both membrane-tethered and soluble forms, thus mediating not only cell recruitment but also adhesion [65]. Evidence has indeed shown that CX3CL1-CX3CR1 interaction mediates firm adhesion of monocytes to smooth muscle cells, which is thought to be at least one mechanism supporting plaque development [66, 67].

Roles of several other chemokine-chemokine receptor axes including CCL20-CCR6, CCL17-CCR4, CXCL1-CXCR2, and CXCL10-CXCR3 in atherosclerosis progression were identified in the mouse models. However, it remains unclear whether these members of the chemokine system, although expressed in atheroma and/or increased in the circulation of hypercholesterolemic and/or CAD patients, contribute to plaque development in humans.

In mice, CCL20 and CCR6 are expressed in healthy vessels and in atherosclerotic plaques [68, 69]. Deletion of *Ccr6* decreased atherosclerosis burden in the *ApoE*$^{-/-}$ mouse, which was accompanied by lower macrophage content in plaques, suggesting that CCR6 is proatherogenic. Moreover, reduced atherosclerosis in *Ccr6*$^{-/-}$ bone marrow chimeras fed atherosclerosis-inducing diet indicates that CCR6^{+} hematopoietic cells contribute to lesion development [70]. This finding is consistent with observations in *Ccr6*$^{-/-}$*Ldlr*$^{-/-}$ mice, which showed that Western diet triggered atherosclerosis in this strain; however, plaque burden in *Ccr6*$^{-/-}$*Ldlr*$^{-/-}$ mice was lower than in the *Ldlr*$^{-/-}$ controls. CCR6 was also shown to promote monocyte adhesion to inflamed endothelium *in vitro* and leukocyte adhesion to carotid arteries *in vivo*. More importantly, CCR6 selectively recruited monocytes but not T cells in an acute inflammatory air pouch model [71]. Thus, because CCR6 controls adhesion and recruitment of monocytes/macrophages to the inflamed vessel, this chemokine receptor may function on multiple levels during plaque development.

CCL17, which is produced by dendritic cells (DCs) and acts through its cognate chemokine receptor CCR4 [72], is upregulated in advanced mouse plaques [73]. Deficiency in *Ccl17* in *ApoE*$^{-/-}$ mice resulted in reduced atherosclerosis that was supported by immunoregulatory functions of Treg cells. Coculture of CD4^{+} T cells with *Ccl17*$^{-/-}$ DCs reduced apoptosis and stimulated expansion of Treg lymphocytes, suggesting that dendritic cell CCL17 is a central regulator of Treg homeostasis [74]. This chemokine may support atherosclerosis progression by inhibiting Treg expansion.

CXCR2 and its chemokine ligand CXCL1 are expressed in mouse plaques [75]. Transplantation of *Cxcr2*$^{-/-}$ bone marrow to *Ldlr*$^{-/-}$ recipients fed Western diet resulted in smaller lesions, suggesting that hematopoietic cell CXCR2 promotes atherogenesis. Similarly, inactivation of *Cxcl1* decreased atherosclerosis burden in *Ldlr*$^{-/-}$ mice [76]. Since

CXCL1 is mainly produced by macrophages, neutrophils, epithelial, and endothelial cells and because CXCR2 is found on neutrophils, monocytes, and mast cells, the proatherogenic effect of CXCL1-CXCR2 axis is likely due to monocyte arrest on atherosclerotic endothelium and CXCR2-mediated macrophage accumulation in lesions [77]. However, since neutrophils were shown to promote atherosclerosis, the CXCR2-CXCL1 axis may also direct neutrophils to developing plaques thereby promoting atherogenesis.

Genetic inactivation of *Cxcr3* or its ligand *Cxcl10*, that were both detected in mouse lesions in the $ApoE^{-/-}$ strain, resulted in decreased atherosclerosis burden that was accompanied by a decrease in inflammatory $CD4^+$ T cell content, an elevated number of Tregs, and increased expression of IL-10 [78]. This finding suggests that the CXCR3-CXCL10 interaction regulates atherosclerosis progression by controlling the recruitment and T cell content in lesions.

Thus, evidence from atherosclerosis-prone mouse models and analysis of human cardiovascular cohorts reinforce the important contribution of multiple chemokines in plaque development.

4.2. Atheroprotective Chemokines and Chemokine Receptors. CCR1, a receptor for CCL3 and CCL5, is expressed on human monocytes, macrophages, and T cells and on mouse neutrophils [79]. Reconstitution of irradiated $Ldlr^{-/-}$ mice with $Ccr1^{-/-}$ bone marrow increased atherosclerotic surface in both the thoracic aorta and the aortic root, indicating that CCR1 opposes atherogenesis [80]. $Ccr1^{-/-}ApoE^{-/-}$ mice have accelerated atherosclerosis relative to $ApoE^{-/-}$ controls. Increased atherosclerosis burden correlated with a significant increase in $CD3^+$ T cell numbers in plaques, whereas lesional macrophage content remained unaffected in these mice [50]. CCR1 was also reported to support CCL5-mediated arrest of monocytes and Th1 cells on activated endothelium [81]. Thus, CCR1 may be protecting from atherosclerosis by preventing excessive T cell and monocyte influx into atherosclerotic vascular wall.

CCR7 promotes homing of T cells and DCs to T cell areas of lymphoid tissues where T cell priming occurs. CCR7 and its ligands CCL9 and CCL21 contribute to a multitude of adaptive immune functions including thymocyte development, secondary lymphoid organogenesis, high affinity antibody responses, regulatory and memory T cell function, and lymphocyte egress from tissues [82]. CCR7 and CCL19/CCL21 are expressed in mouse and human atherosclerotic plaques [83], suggesting that CCR7-CCL19/CCL21 interaction is important in atherogenesis. CCR7 deficiency in $ApoE^{-/-}$ mice exacerbated atherosclerosis, which correlated with a selective increase in circulating T cells and their accumulation in atherosclerotic plaques. $Ccr7^{-/-}ApoE^{-/-}$ mice had increased Th1 and Th17 responses but reduced Th2 and Treg responses [84]. Thus, evidence shows that CCR7 in atherogenic conditions opposes plaque development.

CXCR4 is constitutively expressed on a variety of different cell types. During embryogenesis CXCR4 and its chemokine ligand CXCL12 regulate development of hematopoietic, cerebellar, and endothelial tissues by controlling tissue progenitor cell migration, homing, and survival [85–87]. In adult life, this axis serves as the key factor for physiologic stem cell and immune cell trafficking [88–90]. Plasma CXCL12 levels in CAD patients are lower than in healthy controls [91]. Aged $ApoE^{-/-}$ mice with advanced atherosclerosis have lower levels of CXCL12 in serum and bone marrow than their age matched controls [92], indicating that CXCR4-CXCL12 axis may counter plaque development. Treatment of $ApoE^{-/-}$ mice with the CXCR4 antagonist AMD3465, reconstitution of $ApoE^{-/-}$ mice with $Cxcr4^{-/-}$ bone marrow, or repopulation of $Ldlr^{-/-}$ mice with bone marrow overexpressing "degrakine," which traps CXCR4 in the endoplasmic reticulum, all increased plaque burden. This increase in atherosclerosis burden was associated with neutrophilia and with increased neutrophil content in plaques. More importantly, inhibition of CXCR4 signaling or hematopoietic cell CXCR4 deficiency also coassociated with decreased smooth muscle cell and $CD3^+$ T cell content in plaques, suggesting that lack of CXCR4 may increase plaque vulnerability [93]. Thus, the CXCR4-CXCL12 axis plays a protective role in regulating neutrophil infiltration into the atherosclerotic plaques. Since CXCR4 is conserved across species, understanding how this chemokine receptor functions in the mouse may explain its role in human atherosclerosis.

4.3. Is the Chemokine System Involved in Atherosclerotic Plaque Resolution? Complete resolution of atherosclerosis is a desired clinical goal. However, mechanisms supporting regression of plaques remain obscure. This is due in part to a limited number of preclinical atherosclerosis-prone models in which hypercholesterolemia and vascular inflammation, which are the main drivers of plaque development, can be effectively reversed.

As one approach to this problem, the adenovirus-mediated restoration of apolipoprotein E (ApoE) expression in $ApoE^{-/-}$ mice was shown to reverse hypercholesterolemia and reduce foamy macrophage burden in lesions. The main mechanism supporting regression of plaques in this model is reduced monocyte entry into lesions combined with a stable rate of macrophage apoptosis [94]. If the gene transfer strategy is used to achieve reversal of hypercholesterolemia, regression of atherosclerosis occurs only under sustained, high level ApoE expression, which is yet to be achieved in the clinical setting.

Another model to study atherosclerosis regression involves transplantation of plaque-containing portions of aorta from hypercholesterolemic $ApoE^{-/-}$ mice into normolipidemic C57BL/6 recipients. In this model, normal plasma cholesterol levels in recipients triggered plaque regression that coincided with rapid emigration of inflammatory cells from plaques [95]. The experimental design in this investigation rapidly eliminated hypercholesterolemia and disrupted lymphatic and arterial systems. This approach to normalize plasma lipids does not mimic statin-induced changes in patients and because it is

acute and invasive, it may trigger responses that are stress-induced rather than triggered by normalization of plasma cholesterol thereby limiting the translational potential of this model.

Moderate atherosclerosis regression was observed in the mouse model in which hypercholesterolemia was reduced by switching atherogenic mice fed the Western diet to regular chow diet [96] as well as in hypercholesterolemic mice treated with liver-X-receptor agonist [97] or high-density lipoprotein [98].

Atherosclerotic plaque regression was also observed in the Reversa model ($Ldlr^{-/-}ApoB^{100/100}Mttp^{fl/fl}Mx1\text{-}Cre$, C57BL/6 background) [99, 100]. Reversa mice lack low- density lipoprotein receptor and express atherogenic apolipoprotein B 100. The Western diet-induced hyperlipidemia causes aortic atherosclerosis reminiscent of that in $ApoE^{-/-}$ and $Ldlr^{-/-}$ strains. However, hypercholesterolemia is reversed upon Cre-dependent inactivation of the microsomal triglyceride transfer protein (*Mttp*), which is required for the transport of neutral lipids to nascent ApoB lipoproteins and the assembly of atherogenic LDL in the liver [101]. The Reversa model is ideal for studying regression of atherosclerosis because genetic inactivation of *Mttp* results in moderate and time-dependent lowering of plasma lipids that mimics statin-induced normalization of plasma lipids in patients.

The chemokine system was found to participate in atheroregression. In the transplant model of atherosclerosis regression, the CCR7-CCL19/CCL21 axis was shown to direct emigration of inflammatory cells from plaques, reducing plaque burden and stabilizing lesions [95, 97]. However, the critical role of CCR7 in atheroregression was not confirmed in the model in which overexpression of ApoE was used to reverse hypercholesterolemia [94]. The controversy between these two models remains unexplained; however, it also remains unclear how above normal plasma ApoE levels affect expression and function of CCR7 and/or CCL19/21. This should be investigated because ApoE, in addition to mediating the uptake of chylomicrons, very low-density lipoprotein (VLDL), and their remnants, also activates signaling cascades suppressing production of proinflammatory cytokines, regulates monocyte/macrophage polarity and was demonstrated to play a pivotal role in maintaining the Th1/Th2 balance [102]. Thus, abnormal levels of ApoE may alter expression and function of signals and receptors engaged in atheroregression, thereby inducing mechanisms of plaque regression that may not be physiologically relevant.

5. Conclusion

Given the serious immediate and long-term consequences of the global obesity epidemic, understanding of the mechanisms that support development of obesity and obesity-related comorbidities should be considered a top priority to which is devoted much research interest and effort. Animal experiments have proven indispensable in uncovering mechanisms driving pathophysiology of obesity and atherosclerotic cardiovascular disease. While experimental atherosclerosis and obesity in animals allow the rigorous testing of mechanistic hypotheses, they do not mimic the human condition entirely. Because of this, animal studies require critical evaluation and interpretation and recognition of their limitations. However, animal models, despite being imperfect, have taught us that the pathological mechanisms of obesity recapitulate many features of the inflammatory processes at work in atherosclerosis. Chronic inflammation that drives both obesity and plaque development (Figure 1) is greatly supported by the chemokine system, which makes chemokines and chemokine receptors attractive therapeutic targets for treatment of atherosclerosis or to combat obesity.

Conflict of Interests

The authors declare no conflict of interests or other interests that might be perceived to influence the content of this paper.

Acknowledgments

This work was supported by Grants from the National Center for Research Resources (5 P20 RR018758-08) and the National Institute of General Medical Sciences (8 P20 GM103441-08), from the Oklahoma Center for the Advancement of Science and Technology (HR10-099), and from the Oklahoma Center for Adult Stem Cell Research (4340-03-06 and 4340-04-120). The authors thank Mia Pederson-Rambo for editing the paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 938576, 5 pages
http://dx.doi.org/10.1155/2014/938576

Research Article

Unique Cytokine Signature in the Plasma of Patients with Fibromyalgia

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Received 19 November 2013; Revised 28 January 2014; Accepted 5 February 2014; Published 11 March 2014

Academic Editor: Jong-Young Kwak

Fibromyalgia (FMS) is a chronic pain syndrome with a complex but poorly understood pathogenesis affecting approximately 10 million adults in the United States. The lack of a clear etiology of FMS has limited the effective diagnosis and treatment of this debilitating condition. The objective of this secondary data analysis was to examine plasma cytokine levels in women with FMS using the Bio-Plex Human Cytokine 17-plex Assay. Post hoc analysis of plasma cytokine levels was performed to evaluate patterns that were not specified *a priori*. Upon examination, patients with FMS exhibited a marked reduction in T_H2 cytokines such as IL-4, IL-5, and IL-13. The finding of this pattern of altered cytokine milieu not only supports the role of inflammation in FMS but also may lead to more definitive diagnostic tools for clinicians treating FMS. The T_H2 suppression provides strong evidence of immune dysregulation in patients with FMS.

1. Introduction

Fibromyalgia (FMS) is a chronic pain syndrome in which pathogenesis is complex and cure is not known. It affects approximately 10 million adults in the United States with an estimated 90% of diagnoses being reported in women [1]. The symptom profile of FMS includes pain, fatigue, and distressed mood. Sequelae of FMS include physical and psychological distress, loss of work productivity, reduced quality of life, and increased use of health resources. Annual expenditures for the diagnosis and treatment of FMS are estimated at approximately $20 billion, thus presenting a significant burden to patients, their families, and society [2, 3]. Although the incidence of FMS is rising, the etiology remains unclear. A major theory is that inflammatory mediators lead to complex neuroendocrine aberrations of the hypothalamic-pituitary-adrenal (HPA) axis [4]. Altered levels of cytokines have been associated with symptoms of pain, fatigue, and distressed mood in multiple conditions including painful peripheral neuropathies, hepatitis C, cardiovascular disease, and cancer [3, 5, 6]. This symptom profile mimics the representative symptoms of FMS. Thus, it is theoretically plausible that these nonspecific inflammatory mediators may also contribute to the symptoms of pain, fatigue, and distressed mood in FMS. To date, results of studies examining the association of cytokine alterations with FMS and its symptoms have been mixed [7–9]. Although researchers have suggested FMS as being an inflammatory state related to a dysregulated immune system or altered stress response, the pathophysiological role of cytokines continues to remain unclear [9, 10]. Because there are no diagnostic markers for FMS as well as no identified etiology for the development of FMS, researchers are still searching for mechanistic signs to identify those who already have or those who are at risk for developing fibromyalgia.

T helper lymphocytes are defined by expressing the cell surface molecule known as CD4 and are subdivided further based on the cytokines that they produce. The discovery of the T_H1 and T_H2 paradigm [11] was a pivotal breakthrough in the field of immunology. This balance and counterbalance of inflammatory mediators were delineated and ultimately led to fundamental additions to the knowledge base of cytokine

biology we understand today. Although T_H subsets have expanded far beyond the initial discovery to include T_H17, T_H9, T_{FH} and others probably yet to be discovered, we can still use the T_H1 and T_H2 paradigm to better understand inflammation at both the bench and bedside. T_H1 immune responses are historically associated with antitumor and antiviral responses, whereas T_H2 are associated with humoral immune responses. However, today these designations are being expanded to include other disease states. For example these helper T cell derived cytokines are being examined in disease states such as schizophrenia [12], depression [13], and chronic pain [14].

The purpose of the secondary data analysis in this study was to examine cytokine profiles in women in diagnosed with FMS and to determine if relationships existed among the secreted cytokines detected in the plasma and to determine if any unique cytokine patterns that emerge correlate with disease symptoms.

2. Patients and Methods

2.1. Patients. Two separate studies were conducted: one preliminary and one for validation. Both were approved by the Institutional Review Board of Virginia Commonwealth University. Our preliminary study involved 42 females, whereas the validation study was comprised of 63 females. Inclusion criteria included age ≥18, female, diagnosis of FMS as defined by the 1990 American College of Rheumatology (ACR) criteria, no known major psychiatric or neurological conditions that would interfere with study participation, and an ability to understand and sign the consent form. The 1990 ACR criteria for fibromyalgia require that an individual has both a history of chronic widespread musculoskeletal pain (more than 3 months) and the finding of 11 of 18 possible tender points upon physical examination [15]. Both studies were completed prior to the publication of the 2010 revised FMS diagnostic criteria [16]. Exclusion criteria included presence of other systemic rheumatologic conditions, being immunocompromised (e.g., diagnosis of HIV/AIDs), receiving corticosteroid treatments, being treated for cancer, and/or being pregnant. Self-reported diagnosis of FMS was confirmed by the participant's primary physician or rheumatologist.

2.2. Questionnaires. In both studies, study participants completed self-report form to collect data regarding age, race/ethnicity, marital status, length of time since diagnosis of FMS, socioeconomic status and psychiatric, medical and medication history. Stress was measured using the Perceived Stress Scale (PSS). The 10-item PSS measures the degree to which the individual perceived events in her life over the previous month to be stressful. The scale has an internal reliability of 0.78 and demonstrated construct validity [17]. Pain was measuredusing the Brief Pain Inventory (BPI) Short Form [18]. The BPI assesses pain severity (BPI-S) and pain interference (BPI-I) using 0–10 numeric scales for item rating; higher scores indicate increased pain/interference. Pain severity indicates the intensity of the pain experienced, while pain interference measures the degree to which pain interferes with activities of daily living. In widespread testing, the Cronbach's alpha reliability ranges from 0.71 to 0.91 [19]. Fatigue was measuredusing the Brief Fatigue Inventory (BFI), a simple, 9-item scale that taps into a single dimension of fatigue severity and the interference fatigue creates in daily life. A score of 7 or higher indicates severe fatigue [20]. The BFI has demonstrated excellent reliability in clinical trials, ranging from 0.82 to 0.97 [19]. Depression was measured using the Center for Epidemiological Studies Depression Scale (CES-D). The CES-D is a 20-item self-report instrument comprised of four factors assessing cognitive and affective components of depression. This instrument has very good construct validity, internal consistency, and test-retest reliability [21].

Table 1: Patient demographics.

Number of patients enrolled	$N = 42$
Female	$N = 42$ (100%)
Race	$N = 17$ African American (41.9%)
	$N = 25$ Caucasian (58.1%)
Age	Average = 48.7 (Range 26–75)

2.3. Immunological Assays. Blood samples were collected into heparinized vacutainer tubes for measuring immune markers. Blood was centrifuged for separation of plasma, and all specimens were aliquoted immediately, frozen, and stored at −80° until all samples were collected. All samples were assayed together to reduce interassay variability.

Plasma levels of cytokines such as interleukin (IL) 1beta (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, and TNF as well as chemokines such as CXCL8 (IL-8), CCL2 (MCP1), and CCL4 (MIP1β) were analyzed using the 17-plex Bio-Rad (Bio-Rad; Hercules, CA) cytokine, chemokine, and growth factor assay kit per manufacturer's protocol.

2.4. Data Analysis. All data are presented as the mean +/− standard error of the mean (SEM). All secondary analysis was performed using SigmaPlot software.

3. Results

3.1. Initial Study. The patient demographics can be found in Table 1. Post hoc analysis of plasma cytokine levels was performed to determine if patterns appeared that were not specified *a priori*. Given the fact that the patients were of only two races, analysis was first performed to determine if differences existed in the cytokine levels of Caucasian women with FMS versus African American women with FMS. Using Mann-Whitney U tests, 16 of the 17 cytokines assayed displayed no statistical difference among race (data not shown) and were thus used for further analysis. Power analysis was performed to ensure a 95% confidence level with a confidence interval of 15%. Results from this analysis indicated that an appropriate N for cytokines would be equal to or greater than 21. We then eliminated three more

Table 2: Cytokine measured.

Cytokine/chemokine	Average ± SEM	Normal range
CCL2	38.0 ± 3.1	50.0–298.8
CXCL8	14.5 ± 10.8	6.4–20.4
IL-1β	4.3 ± 3.4	0.0–1.2
IL-4	0.6 ± 0.1	5.5–12.5
IL-5	1.2 ± 0.1	6.0–44.5
IL-6	9.1 ± 2.9	11.0–17.0
IL-7	9.5 ± 0.8	0.0–22.0
IL-12p70	5.7 ± 0.6	1.7–2.5
IL-13	2.8 ± 0.3	12.0–47.9
GCSF	89.8 ± 6.1	0.0–34.4
GMCSF	26.9 ± 5.9	2.0–48.0
IFNγ	32.9 ± 6.5	1.2–25.0
TNF	16.4 ± 2.1	2.1–46.0

Table 3: Patient demographic.

Number of patients enrolled	$N = 63$
Female	$N = 63$ (100%)
Race	$N = 18$ African American (28.6%)
	$N = 45$ Caucasian (71.4%)
Age	Average = 47 (Range 18–71)

cytokines for further analysis given that the N of patients with detectable levels was below 21. These stringency criteria left 13 cytokines and chemokines for further examination. The values for our FMS patients are presented in Table 2.

Due to the fact that at study initiation normal controls were not collected, we used plasma values reported in the literature [22–30]. While we acknowledge this weakness, we are confident that the analyses are still powerful and will warrant further examination into cytokine deviations present in FMS. In order to move forward in the study, we only looked at cytokines that were at least 2X greater in difference than 2SEMs of the mean. Thus, IL-4, IL-5, IL-13, and GCSF were further examined. Upon further scrutiny of the remaining 4 cytokines, a stark pattern began to emerge in that these cytokines are associated with T_H2 immunity.

3.2. Validation Study. To confirm these findings, a secondary set of FMS were analyzed. These patients were recruited for a different study and thus were completely independent of the subjects enrolled in the initial study. The patient demographic can be found in Table 3. Given the drastic differences observed in the original data set for IL-4, IL-5, IL-13, and GCSF, these cytokines were further scrutinized in the independent validation data set. Using the aforementioned criteria, we confirmed that IL-4, IL-5, and IL-13 are indeed suppressed in patients with FMS (Table 4).

3.3. Correlation Analysis. Upon the observation that patients with FMS had suppression in T_H2 cytokines, we began to explore the potential relationship between T_H2 immunity and the psychometrics obtained from the individuals. While

Table 4: Cytokines validated.

Cytokine/chemokine	Average ± SEM
IL-4	1.8 ± 1.1
IL-5	1.0 ± 0.4
IL-13	4.4 ± 1.4
GCSF	12.4 ± 2.4

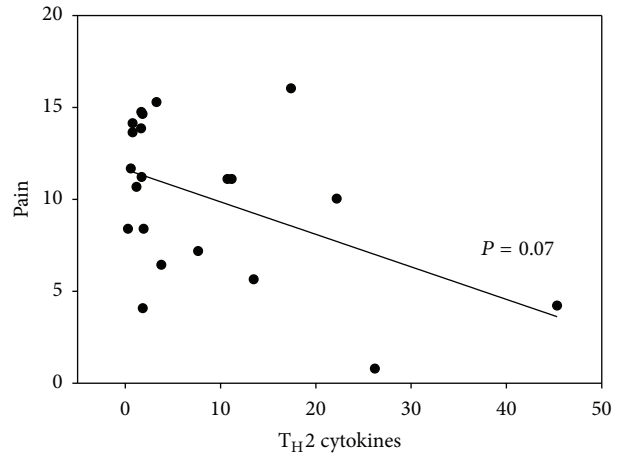

Figure 1: Correlation of pain and T_H2 cytokines. A total T_H2 value was determined by adding plasma values of IL-4, IL-5, and IL-13. A total pain value was determined adding BPI-I and BPI-S using the Brief Pain Inventory scale as described in the material and methods. A Spearman's Correlation was performed on the two values using SigmaPlot and data is shown. Correlation coefficient −0.391, P value 0.07.

no correlations proved significant among cytokine levels and fatigue, depression, or stress (data not shown), there is a trend towards significance when we compared T_H2 cytokine levels and pain ($P = 0.07$, Spearman's) as shown in Figure 1.

4. Discussion

Although not classified as an immune disease by nature, our group as well as others has reported cytokine and immune alterations in patients with FMS [3, 7, 9, 15, 23]. Given the fact that disease etiology is still uncertain, further research is needed in the field to help uncover exact disease pathology in hopes to provide better therapeutic options the millions of FMS patients worldwide. The purpose of this analysis was to examine cytokine alterations in patients with FMS that were not determined *a priori*. Comparing the observed cytokines in the plasma of these patients we noticed a stark decrease in the amount of T_H2 cytokines produced (IL-4, IL-5, and IL-13) as those values reported by other groups in the literature. We extended our analyses to include a secondary, independent data set and once again this unique cytokine signature was observed. To examine a potential underlying cause for the T_H2 suppression, we then correlated cytokine levels to pain, stress, fatigue, and depression which are all symptoms shared in the FMS spectrum. When pain and T_H2 levels were compared we observed a trend that

approached statistical significance. Interleukin 4, the classic T_H2 cytokine has been shown to have both anti-inflammatory and analgesic properties in murine models of mechanical [31] as well as having lower gene expression and serum levels in patients with widespread pain syndromes [6]. IL-13 exhibits analgesic properties in a murine model of *L. major* infection [32], whereas little is reported in regard to IL-5's ability to combat pain. Thus, our preliminary findings suggest that further research into the T_H1-T_H2 imbalance in FMS and its implication in pain are certainly warranted.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

Components of the research were supported by the National Institute of Nursing Research through Grant no. P20 NR008988 (N. McCain, PI) and Grant no. P30 NR011403 (MJ Grap, PI).

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