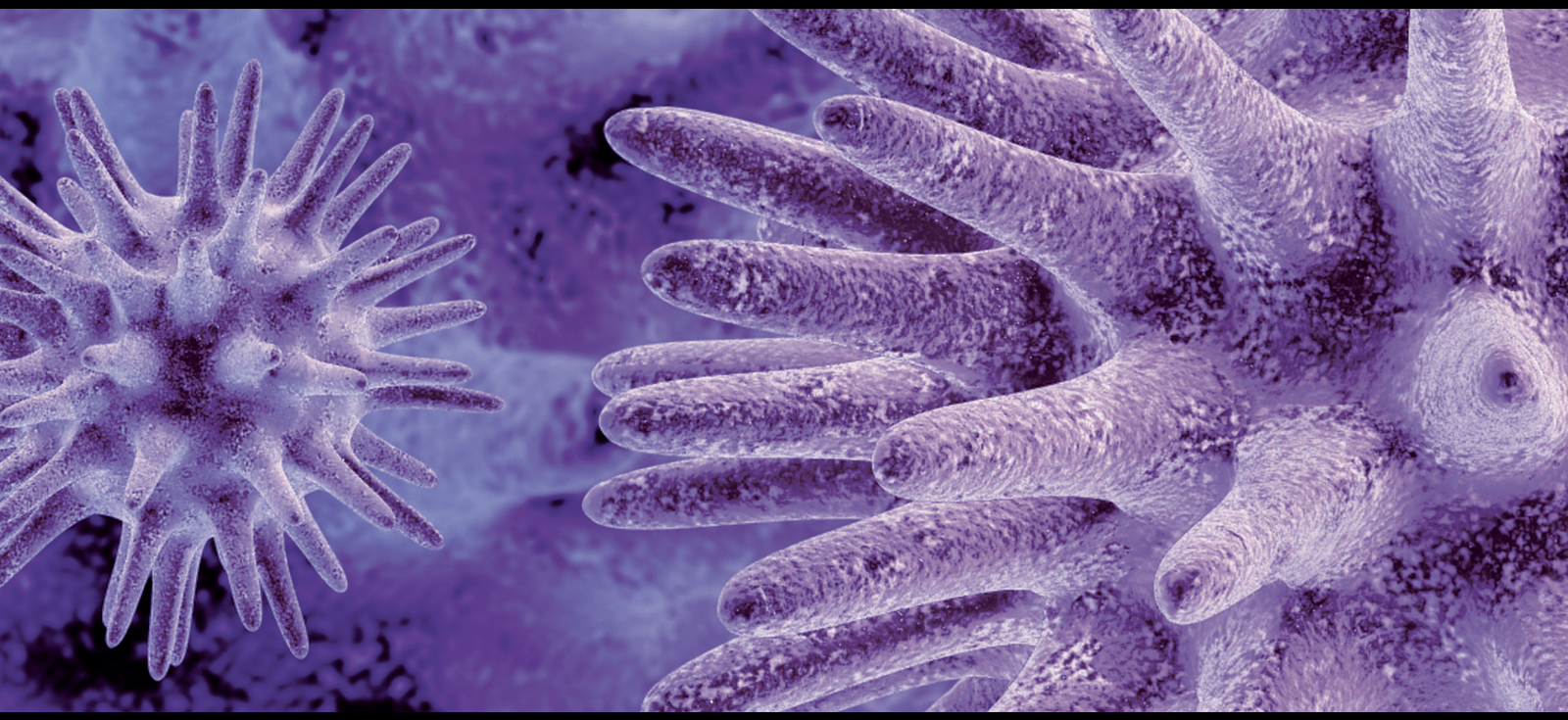
Clinical and Developmental Immunology

Clinical and Experimental Immunomodulation

Guest Editors: Lenin Pavón, Hugo Besedosky, Oscar Bottasso, Rogelio Hernández, Marco Velasco, and Roger Loria



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Editorial Board

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Editorial

Clinical and Experimental Immunomodulation

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The inflammatory response is modulated by the concentration of soluble mediators and the coordinated action of different types of immune cells. Furthermore, basic and clinical research has demonstrated that the immune response is regulated by several factors such as: the chemical nature and the concentration of antigen; the route of administration; the cell type involved in the antigen presentation to their specific lymphocytes; and the presence of antibodies and/or immune complexes among other mechanisms.

More recently, it has been described that other signaling molecules like neurotransmitters and hormones can also modulate the immune response. Over time, this information has enabled the elucidation of the role of immune cell products in physiological processes like sleep, memory, learning, and pain, or in autoimmune and infective diseases, as well as the mechanisms involved. Such evidence provides the opportunity for the development of novel therapeutic approaches for diseases with deleterious immune and inflammatory components. The papers presented in this special issue focus on the leveraging knowledge of clinical and experimental immunomodulation.

First, the reader can find seven experimental approaches that analyze immunomodulation mediated by hormones, neurotransmitters, cytokines, and antigens. The work of M. V. Legorreta-Haquet et al. shows that prolactin in early stages of B cells maturation process may promote the survival of self-reactive clones in a murine model of lupus. T. Schaumann et al. present results of anti-inflammatory effects of glycine in gingival inflammation and encourage further research on the utility of glycine in the prevention therapy of inflammatory periodontitis. B. Dénes et al. share an interesting work on experimental immunotherapy with a multicomponent vaccine containing a cholera toxin B subunit-autoantigen fusion protein for restoration of euglycemia and immunological homeostasis in NOD mice. F. Robledo-Ávila et al. explored a novel therapeutic approach consisting in the administration of murine dialyzable leukocyte extracts plus a reduced, and therefore less toxic, dose of Amphotericin B in a mouse model of systemic candidiasis. The approach proved to be effective in reducing mortality, pathogen burden, and tissue damage at the renal level. S. Mburu et al. evaluated *in vitro* the modulation of LPS-induced CD4+ T cell activation and apoptosis by antioxidants in cells from untreated asymptomatic HIV infected participants. Their results set the basis for the development of an adjuvant therapy aimed to counteract the harmful effects of chronic immune activation on CD4+ T cells. S. Dang et al. show that LMW-HA modulates papillary thyroid carcinoma (PTC)

cell behavior via TLR-4 signaling providing examples of the functional roles of CXCR7 in proliferation and migration. Their data are elegantly complemented with the analysis of TLR4 and CXCR7 expression in PTC clinical samples. Finally, J. M. Calleja-Castillo et al. investigate the effect of deep brain stimulation (DBS) at hypothalamic nucleus in Wistar rats, over the circulating concentrations of corticosterone and proinflammatory cytokines, detecting that the chronic application of this therapy to Wistar rats induces a significant circulatory rise in inflammatory mediators and blocks HPA axis activity. These results suggest that immunity might be altered in patients who are treated with DBS and provide the basis for the development of strategies to prevent immunity-related secondary effects of DBS.

Regarding the clinical approaches of immunomodulation, three works are also included. The first one, from N. Valero-Pacheco et al., analyzes the expression of PD-L1 on T cells in patients infected with the influenza virus A(H1N1)pdm09 and its impact on T cell responses. The second one, from J. Galicia-Carreón et al., studies the context of the unbalanced immunological mechanisms underlying the development of allergic conjunctivitis by evaluating the frequency of Tregs as well as cells expressing homing receptors in peripheral blood from patients. The third one, from M. E. Hernández et al., presents the results of a clinical followup of major depressive disorder (MDD) patients treated with a combination of selective serotonin reuptake inhibitors (SSRI) and human dialyzable leukocytes extract (hDLE) as immunomodulator. The latter consists of small weight peptides and has been used successfully as adjuvant therapy in diverse infectious and deficient cell-immunity problems. MDD patients present imbalances in neurotransmitter levels, hormones such as cortisol, and cytokines that contribute to the behavioral and immune disturbances observed in them. This combined treatment efficiently restored the pro- and anti-inflammatory cytokine balance and cortisol levels when compared with patients treated only with SSRI. This study constitutes the first report of a clinical assay that analyzes the effects of immunotherapy in MDD.

This special issue also includes two reports of experimental techniques that allow the assessment of immunomodulation. The work of I. Lima Siman et al. evaluated the serum levels of allergen-specific IgG antibodies from atopic patients. The authors conclude that this laboratory test would help specialists to follow up patients under immunotherapy. The report by M. C. Jiménez-Martínez et al. shows an experimental technique to identify NnTreg lymphocytes by staining them with *Amaranthus leucocarpus* lectin and posterior FACS.

Last but not least, this issue presents four revisions on a broad range of topics. N. Deckx et al. focus on multiple sclerosis and discuss the influence of neuroendocrine immune system over the susceptibility and severity of autoimmune diseases, as well as new therapeutic approaches for the treatment of this kind of diseases. R. Cabezón and D. Benitez-Ríbas review the participation of different dendritic cells (DCs) subsets and their role in inflammatory bowel disease and present preclinical studies performed in animal models describing the recent characterization of tol-DCs from Crohn's disease patients. G. A. Toledo-Ibarra et al. describe some aspects of the immunity of fish and its connections with cholinergic system, highlighting the possibility that bidirectional communication between the nervous and immune systems exists in lower vertebrates as well as during evolution of immune system. G. Hurtado-Alvarado et al. present an extensive review focused on the relationship between inflammation and inflammatory markers as well as sleep and sleep loss.

Acknowledgment

We wish to thank the authors and reviewers for their contributions to this special issue. We hope that our readers will find this special issue enticing and enjoy reading contributions by all authors, as we have done.

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Review Article

Neuroendocrine Immunoregulation in Multiple Sclerosis

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Currently, it is generally accepted that multiple sclerosis (MS) is a complex multifactorial disease involving genetic and environmental factors affecting the autoreactive immune responses that lead to damage of myelin. In this respect, intrinsic or extrinsic factors such as emotional, psychological, traumatic, or inflammatory stress as well as a variety of other lifestyle interventions can influence the neuroendocrine system. On its turn, it has been demonstrated that the neuroendocrine system has immunomodulatory potential. Moreover, the neuroendocrine and immune systems communicate bidirectionally via shared receptors and shared messenger molecules, variously called hormones, neurotransmitters, or cytokines. Discrepancies at any level can therefore lead to changes in susceptibility and to severity of several autoimmune and inflammatory diseases. Here we provide an overview of the complex system of crosstalk between the neuroendocrine and immune system as well as reported dysfunctions involved in the pathogenesis of autoimmunity, including MS. Finally, possible strategies to intervene with the neuroendocrine-immune system for MS patient management will be discussed. Ultimately, a better understanding of the interactions between the neuroendocrine system and the immune system can open up new therapeutic approaches for the treatment of MS as well as other autoimmune diseases.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS). It is characterized by inflammation, demyelination, axonal degeneration, and gliosis. MS affects 1 out of 1000 people in the Western world and leads to chronic disability in mostly young adults (20–40 years). This neurodegenerative disease is characterized by a heterogeneous clinical course with motor sensory and sensible disturbances [1]. The majority of patients (85%–90%) starts with relapses followed by remissions (i.e., relapsing-remitting (RR)-MS). Relapses are a defining feature of MS and reflect focal inflammatory events. With time and age, most patients switch to a progressive phase with gradual deterioration of neurological functions due to progressive axonal degeneration (i.e., secondary progressive (SP)-MS). About 10%–15% of MS patients are diagnosed with primary progressive MS (PP-MS). This progressive form is characterized by a gradual clinical decline in functions with no distinct remissions.

Although MS is considered to be a predominantly immune-mediated demyelinating disease, as demonstrated by immune cell infiltration and accompanying inflammatory processes leading to damage of myelin, the etiology of MS is unknown. It is now generally accepted that MS is a complex multifactorial disease involving genetic and environmental factors affecting the autoreactive immune responses [2]. In this respect, we will address here the role of the neuroendocrine system in MS. Several studies have addressed the possible role of the neuroendocrine system in susceptibility and severity of autoimmune diseases. Moreover, it has been shown that the neuroendocrine system has immunemodulatory potential [3]. Ultimately, a better understanding of the interactions between the neuroendocrine system and

the immune system can open up new therapeutic approaches for the treatment of autoimmune diseases, including MS.

2. The Neuroendocrine-Immune System

The neuroendocrine system is based on interactions between the nervous and the endocrine system. Furthermore, the neuroendocrine system can both directly and indirectly influence the developmental and functional activity of the immune system. In turn, the immune system can collaborate in the regulation of endocrine activity. The bidirectional interactions between aforementioned systems are known as the neuroendocrine-immune system. The integration between these two systems is essential in order to maintain homeostasis and health. Neuroendocrine regulation of immune responses is important for survival during both physiological and mental stress. Systemically, this regulation is accomplished by hormones, such as those from the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-pituitary-gonadal (HPG) axis. Regional regulation is accomplished by innervations, including the autonomic nervous system, while local regulation is accomplished by neurotransmitters [4]. The immune system regulates the CNS through immune mediators and cytokines that can cross the blood-brain barrier (BBB), or signal indirectly through the vagus nerve or second messengers. Furthermore, an entire constellation of neurotransmitters and neuroendocrine hormones is known to be endogenously produced by the immune system, while the hypothalamus and/or anterior pituitary have been shown to express interleukin (IL)-1, IL-6, transforming growth factor (TGF)-β, and other cytokines. Additionally, immune, endocrine, and neural cells express receptors for hormones, cytokines, and neurotransmitters. Hence, these products act in an autocrine, paracrine, and endocrine manner thereby further supporting the postulated bidirectional interactions of the neuroendocrine-immune system [5]. In summary, the neuroendocrine and immune systems communicate bidirectionally via shared receptors and shared messenger molecules, variously called hormones, neurotransmitters, or cytokines.

3. Regulation of the Immune System by the Neuroendocrine System and Dysfunction in MS

In a healthy individual, the neuroendocrine and the immune system provide a finely tuned regulatory system. Disturbances of these regulatory systems could potentially lead to oversuppression of the immune system for example, resulting in a higher susceptibility to cancer and infectious diseases, or overactivation of the immune system which on its turn may lead to a higher risk for inflammatory or autoimmune diseases.

3.1. The Hypothalamic-Pituitary-Adrenal (HPA) Axis. In order to survive, organisms maintain a complex dynamic equilibrium or homeostasis which is constantly challenged by intrinsic or extrinsic factors such as emotional, psychological, traumatic, or inflammatory stress. For several decades, it has been known that the hormonal stress response is mainly coordinated by the HPA axis. The HPA axis is a regulatory system, including the hypothalamus, pituitary, and adrenal glands and regulatory neural inputs, which functions on both a neuronal and an endocrine level through the release of neural factors and hormones. It has central and peripheral actions, mediates the coordination of circadian events such as the sleep/wake cycle, and helps with coping, adaptation, and recovery from stress.

During various physical and psychological stimuli, the HPA axis is activated which results in secretion of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus (PVN) of the hypothalamus into the hypophyseal portal blood supply. CRH acts on the anterior pituitary gland to stimulate the release of adrenocorticotropic hormone (ACTH). Subsequently, ACTH circulates through the systemic circulation towards the adrenal cortex where it induces the expression and release of glucocorticoids (GC) in a diurnal pattern (Figure 1). The secretion of CRH is upregulated by serotonergic [6], cholinergic [7], and catecholaminergic systems [8]. On the other hand, opiates and γ-aminobutyric acid (GABA) as well as hormones downstream of CRH, such as GC and ACTH, can inhibit the secretion of CRH via negative feedback [9].

It is known that GC, which are amongst the best-characterized hormones, exert a wide variety of immunomodulatory effects, including modulation of cytokine expression, cell adhesion and migration, and production of inflammatory mediators [10, 11]. The immunomodulatory effects of GC are regulated through intracellular glucocorticoid receptors which have a widespread distribution throughout various tissues. There are two different types of glucocorticoid receptors including the high affinity type 1 mineralocorticoid receptor (MR) which mediates non-stress-related circadian fluctuations in GC and is primarily activational. In contrast, the low affinity glucocorticoid receptor (GR) mediates stress levels of GC and is inhibitory in some systems, while being activational in others [12]. Although GC are generally immunosuppressive at pharmacological concentrations, GC are immunomodulatory at physiological levels. Upon ligation, the transcription of target genes is directly and/or indirectly affected by binding of the GR to specific sequences of DNA, known as GC-responsive elements (GRE). In this perspective, GC specifically regulate the immune response causing a shift from T helper type 1 (Th1) to Th2 immune responses. Indeed, GC directly inhibit the production of pro-inflammatory cytokines, such as IL-1, IL-6 and Th1-related cytokines (IL-2, IL-12, and IFN-γ) as well as inflammatory mediators, such as prostaglandin and nitric oxide [10], while GC increase the production of anti-inflammatory Th2-related cytokines (IL-4 and IL-10). In doing so, GC enhance immunoglobulin production [13, 14]. Besides, GC have a direct inhibitory effect on the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin. These adhesion molecules play a key role in the trafficking of inflammatory cells to sites of inflammation [15]. Furthermore, GC negatively affect dendritic cells

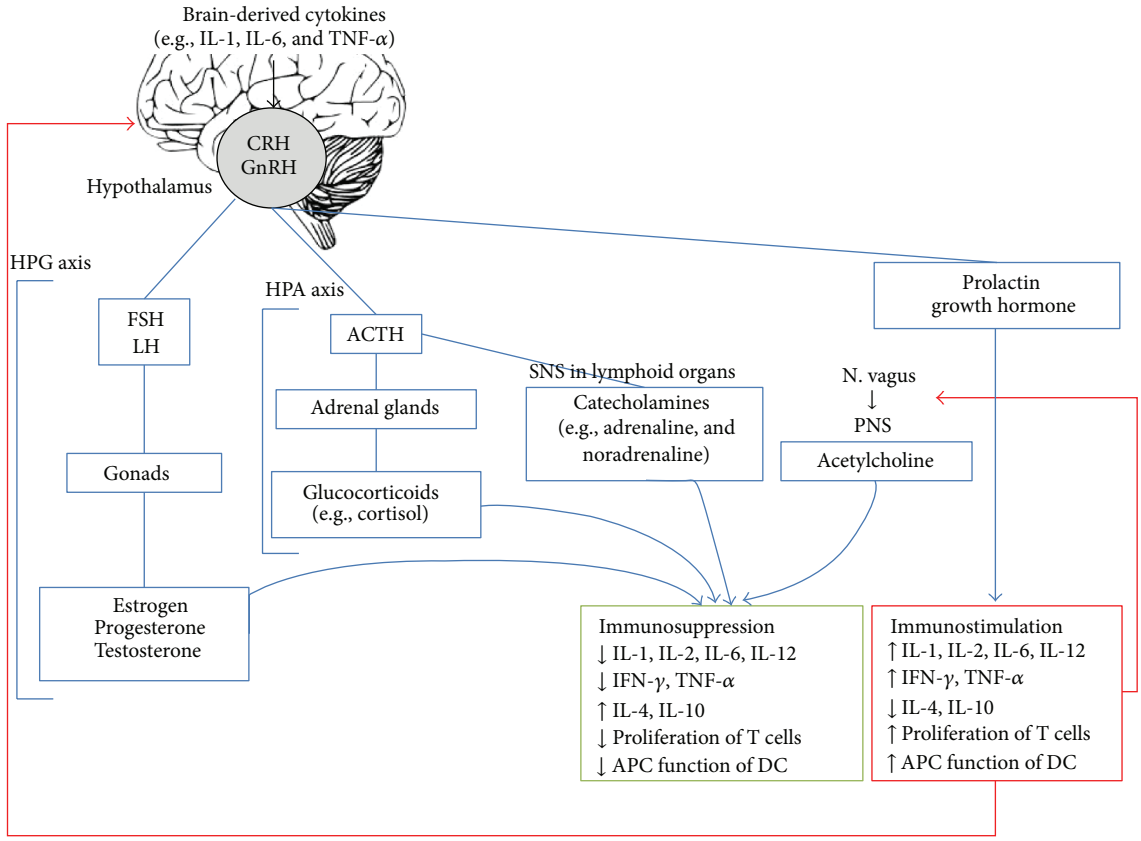

Figure 1: The neuroendocrine-immune system. Via a complex system of common messenger molecules and receptors, the neuroendocrine and the immune systems bidirectionally communicate and monitor each other's activities. Integration of these signals is essential to maintain homeostasis and health and may result in immunosuppression or immunostimulation. Discrepancies at any level can lead to changes in susceptibility to and severity of several autoimmune and inflammatory diseases.

(DC), the most specialized antigen-presenting cells (APC), by suppressing their maturation and by downregulating the expression of the major histocompatibility complex (MHC) molecules [16]. On the other hand, GC can indirectly suppress immune responses through the inhibition of pro-inflammatory transcription factors such as nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) [17] and activating protein-1 (AP-1) [18]. NF-κB promotes the expression of the genes coding for many cytokines, enzymes, and adhesion molecules involved in inflammatory diseases [19]. Hence, also the inhibition of the activation of NF-κB contributes to the anti-inflammatory actions of GC.

A well-known GC is cortisol, often referred to as the stress hormone and a powerful natural immunosuppressor. Following binding to glucocorticoid receptors, cortisol is involved in several regulatory functions such as glucose metabolism, regulation of blood pressure, insulin release for blood sugar maintenance, immune function, and inflammatory responses. For example, studies have shown that cortisol can prevent T cell proliferation by downregulation of the IL-2 receptor [20]. During the body's fight or flight response to stress, cortisol is secreted at higher levels and is responsible for several stress-related changes in the body. Moreover, this immunosuppressive hormone plays an important role in the circadian rhythm as its plasma levels exhibit a diurnal pattern with peak levels in the morning at approximately 9 am and a nadir at night [21]. Interestingly, some cytokine concentrations also follow a diurnal rhythm. Proinflammatory mediators in serum, such as IL-1, IL-6, and soluble IL-2 receptors, peak at 1–4 am and are low throughout the day with a nadir at 8–10 am when cortisol levels are the highest [22–24]. Interestingly, circadian involvement has been noted in various autoimmune and inflammatory diseases [25]. Indeed, Cutolo et al. have documented that clinical signs and symptoms of patients with rheumatoid arthritis (RA) vary within a day [25]. More severe symptoms are often presented upon waking in the morning possibly associated with peak levels of pro-inflammatory cytokines during the night. Melatonin, which antagonizes the immunosuppressive effects of cortisol, is secreted by the pineal gland in the brain. Melatonin levels begin to rise in the midevening to late evening, peak at approximately 3 am, and then drop in the early morning hours. It has been demonstrated that melatonin production in RA patients is increased in comparison with healthy controls

at the beginning of the night and in the early morning and is correlated with the typical peak of joint stiffness and pain.

Clinical and experimental studies have demonstrated that abnormalities in the HPA axis in MS may contribute to enhanced susceptibility to disease and to more severe disease activity [26–28]. Although experimental data in experimental autoimmune encephalomyelitis (EAE), the most commonly used animal model of MS, have suggested low reactivity of the HPA axis as a predisposing factor for disease susceptibility and severity [29, 30], it has been demonstrated that up to 50% of MS patients are endowed with HPA axis hyperactivity [31]. Basal plasma levels of cortisol and ACTH were found to be elevated [32] and adrenal glands were demonstrated to be enlarged in MS patients [33]. It was shown that after CRH stimulation, the cortisol response varied according to the disease status of the MS patient and was lower in SP-MS patients compared to patients with PP-MS and healthy controls, while a higher β-endorphin/ACTH response was found in RR-MS patients as compared to other groups [34]. Moreover, higher cortisol levels were often determined during or in close proximity to acute relapse, which is characterized by an MRI-confirmed inflammatory state [26, 27, 34, 35] and correlated with higher white blood cell counts in the cerebrospinal fluid (CSF) [26]. In addition, histopathological findings of the hypothalamus reveal perturbations in CRH regulation as a result of MS lesions in this area. Indeed, an elevated number and activity of CRH-immunoreactive neurons co-expressing vasopressin (i.e., CRH/VP neurons) were found in the hypothalamus of MS patients compared to controls in postmortem studies [33, 36, 37]. Whereas these observations were confirmed by Huitinga and colleagues, they additionally reported an inverse correlation between active MS lesions and the number of hyperactive CRH/VP neurons and levels of CRH mRNA [28]. Furthermore, they observed a more severe disease course in MS patients with high active lesions in the hypothalamus and the lowest CRH expression, suggesting impaired cortisol secretion and reduced ability to control inflammation. The authors hypothesized that this effect was mediated by APC present in the active lesion suppressing the CRH/VP neurons thereby contributing to a more severe disease. Noteworthy, CRH mRNA levels return to normal during remission [29].

Altogether, the HPA axis hyperactivity in MS has been accompanied with progressive disease and global neurodegeneration [38]. Experimental studies suggest that stress and excessive levels of GC may contribute to cellular and molecular disturbances in the brain which may lead to damage in several brain areas including the hippocampus. Indeed, Gold et al. observed smaller hippocampal volumes in MS patients as compared to healthy controls [39]. Given the important role of the hippocampus in learning, mood regulations, memory, and the HPA axis control, as well as the notion that fatigue and depression are among the most common symptoms of MS, significant associations between HPA axis activity and depressive symptoms have been observed in RR-MS during relapse [26]. Gold et al. have detected normal morning but increased evening cortisol levels in MS patients with depressive symptoms compared to non-depressed MS patients [40] as well as compared to age- and gender-matched healthy controls [39]. Although overall RR-MS patients expressed a significantly higher cortisol awakening response compared to healthy controls, only RR-MS patients with moderately elevated depression scores showed significant differences in their cortisol release, while RR-MS patients with low depression scores expressed similar circadian patterns as healthy controls [41].

Besides the release of GC including cortisol, the HPA axis also regulates the secretion of prolactin and growth hormone (GH). Accordingly, these hormones exhibit immunoregulatory effects. Briefly, through stimulation by suckling and stress, prolactin is released from the anterior pituitary gland and stimulates mammary growth and differentiation. Moreover, it is documented that prolactin has immunostimulatory effects such as increasing the production of IFN-γ and IL-12 and the proliferation of T cells [42, 43]. On the other hand, GH mediates its effect through insulin-like growth factor-1 (IGF-1) [44]. Both GH and IGF-1 modulate the immune system by inducing the survival and proliferation of lymphoid cells [45]. In addition to these well-described effects on adaptive immunity, prolactin and GH also modulate innate immunity. Indeed, both hormones enhance activation of macrophages and induce subsequent release of reactive oxygen species (ROS) [46, 47].

3.2. The Hypothalamic-Pituitary-Gonadal (HPG) Axis. In addition to the HPA axis, other central hormonal systems, such as the HPG axis, modulate the immune system [48]. To date, it is generally accepted that gender affects the susceptibility and course of autoimmune diseases. Whereas almost 8% of the world population develops an autoimmune disease, approximately 78% of them are women. Also, MS has a higher prevalence, but better prognosis in women than in men [49].

The integrating center of this reproductive hormonal axis is the hypothalamus. Gonadotropin-releasing hormone (GnRH) is synthesized and released by the hypothalamus into the hypophyseal-portal circulation. Upon transport to the pituitary gland, GnRH stimulates the synthesis and secretion of gonadotropic hormones including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which following systemic release circulate towards the reproductive organs and subsequently stimulate the release of estrogen and progesterone.

Estrogen is a potent steroid with pleiotropic effects and is present in high levels in females from adolescence to menopause. There are 3 naturally occurring estrogens: estrone (E1), estradiol (E2), and estriol (E3) which are the predominant forms during menopause, in non-pregnant females, and during late pregnancy, respectively. Estriol has been accepted as the safest of the three and has been used worldwide for the treatment of menopausal symptoms [50, 51]. Estrogen exerts its effect through binding to two forms of nuclear estrogen receptors (ER), ERα and ERβ, which exhibit distinct transcriptional properties. ERα is expressed on the endometrium, ovarian stromal cells, breast, and hypothalamus, whereas ERβ is widely expressed in tissues including brain, kidney, bone, heart, lungs, intestine, and

endothelial cells [52]. In addition, expression of ER has been demonstrated in a variety of immune cells including monocytes, neutrophils, DC, T cells, and B cells, thereby providing indirect evidence for its immunomodulatory properties [53, 54]. Following ligation, the ER interacts with the transcription factor NF-κB, thereby affecting secretion of cytokines, chemokines, and matrix metalloproteinase (MMP)-9, as well as antigen presentation and function of DC [55]. More specifically, pretreatment with 17β-estradiol blocked the ability of DC to present antigen to T cells resulting in an altered pattern of cytokine production, as evidenced by an increase of Th2 cytokines, such as IL-10 and IL-4, and a decrease of Th1 cytokines including TNF-α and IFN-γ. Furthermore, it was shown that 17β-estradiol treatment significantly decreased the frequency of DC migrating towards the CNS at the onset of EAE [56, 57]. Likewise, estriol decreased the secretion of MMP-9 by immune cells thereby abrogating subsequent migration of inflammatory cells towards the CNS [58]. This effect may be indirectly mediated through downregulation of TNF-α [59], which activates MMP-9 [60]. In addition to their anti-inflammatory effects, estrogens also appear to be neuroprotective in several CNS disorders such as MS, Alzheimer's disease, Parkinson's disease, and spinal cord injury [61–64], as evidenced by improvement of clinical disease and reduction of neuropathology following estrogen treatment. Reported neuroprotective effects are inhibition of neuronal loss by decreasing glutamate-induced apoptosis [65] and protection of oligodendrocytes from cytotoxicity [66] as well as stimulation of oligodendrocyte function [67] and upregulation of TGF-β production from astrocytes [68].

Furthermore, pregnancy, postpartum period, and menopause as well as other physiological conditions have been demonstrated to affect the clinical course of a variety of autoimmune disorders. These clinical observations suggest the importance of sex hormones in immune modulation. Several studies have documented that, during pregnancy, both clinical symptoms and relapse rate of MS are decreased, whereas the postpartum period is associated with a higher risk for exacerbation of the disease [69, 70]. This suggests a role for the hormones fluctuating at this time, such as, cortisol, progesterone, and estrogen, in the regulation of MS activity [71]. It may be clear that pregnancy induces changes in the maternal immune system in order to protect the foetus. The increase of estrogen, progesterone, and cortisol, during pregnancy is associated with increased production of Th2 cytokines and decreased production of Th1 cytokines. Hence, the improvement of MS symptoms during pregnancy may be linked to a shift from the prevailing Th1 response to a Th2 response, while postpartum worsening may be associated with the return to the Th1 environment [72]. The improvement of symptoms occurs predominantly during the third trimester of pregnancy when circulating estrogen and progesterone levels peak, while the postpartum period is characterized by an abrupt drop in estrogen levels. It needs to be noted that, consistent with these findings, hyperestrogenic states are associated with disease flareup of systemic lupus erythematosus (SLE) in which Th2-mediated humoral response is an important pathogenic factor [73].

Sex differences have also been observed in EAE. Female mice are more susceptible to EAE than males, albeit that a genetic background may also influence the effects of sex hormones on the immune system [74]. Interestingly, the minimal effective estrogen dose that inhibits EAE varies greatly between mouse strains [75] suggesting that estrogen receptor sensitivity may influence MS risk. In addition, it has been reported that ERα ligand treatment can ameliorate EAE by decreasing pro-inflammatory cytokines, such as TNF-α and IFN-γ, while enhancing the secretion of the anti-inflammatory cytokine IL-5. Furthermore, reduced CNS white matter inflammation, protection against axonal loss, and demyelination in EAE were documented [76].

In summary, the numerous immunomodulatory and neuroprotective effects of estrogens can attribute to their protection in several neurodegenerative and autoimmune diseases. Next to estrogens, other hormones released through the HPG axis exert immunoregulatory effects. Briefly, high levels of prolactin have been described in MS patients [77], resulting in increased production of IFN-γ and IL-2 by Th1 cells and autoantibody production through activation of Th2 cells [78]. In addition, testosterone inhibits both innate and adaptive immunity. It has been reported that testosterone can enhance production of IL-5 and IL-10 and decrease IFN-γ production by T cells *in vitro* thereby promoting a Th2 response [79]. Treatment with androgen significantly delayed onset and progression of EAE [80–82]. The protective effects of androgens were accompanied with decreased production of Th1 cytokines [82] and increased production of anti-inflammatory IL-10 [80] as well as inhibition of T cell infiltration into the spinal cord [83]. The protective effects of androgens were further confirmed by the observation that, in human male MS patients, low testosterone levels but higher estradiol levels are associated with a higher degree of brain tissue damage [84]. On the other hand, progesterone exerts anti-inflammatory effects by inhibiting NF-κB and enhancing IL-4 production [85, 86]. In EAE, progesterone treatment results in a decreased production of inflammatory IL-2 and IL-17 and an increased production of IL-10 resulting in attenuated disease severity [87]. Furthermore, progesterone enhanced axonal density and reduced axonal damage in EAE [88].

3.3. Neuronal Pathways

3.3.1. Regional Regulation by Innervations. Regional regulation of the immune system through the autonomic nervous system is mediated by innervations of primary and secondary lymphoid organs. Furthermore, T cells, B cells, and DC are located adjacent to nerve terminals. Depending on the pathological conditions, innervation of lymphoid organs can change. For example, the number of innervations in lymphoid organs increases under psychosocial stress in primates, whereas it decreases following viral infection [89].

(1) *Sympathetic Nervous System.* The catecholamines, adrenalin and noradrenalin, are released from sympathetic nerve terminals upon stimulation. Stress situations, such as a physical threat, excitement, a loud noise, or a bright

light, are the major physiological triggers of the release of catecholamines. These stimuli are processed by the CNS through release of ACTH. Subsequently, ACTH stimulates the synthesis of adrenalin and noradrenalin both directly as well as indirectly via cortisol production. Through the release of catecholamines in lymphoid organs, the sympathetic nervous system (SNS) has been demonstrated to exert a direct role in immunomodulation. Whereas most studies have demonstrated that activation of the SNS inhibits the immune system, some studies show opposite effects including induction of chemokines [90]. This possible paradigm can be explained by various actions of adrenalin and noradrenalin through ligation of different receptors. Indeed, the stimulation of α-adrenoreceptors (α1AR) is predominantly associated with immunostimulatory effects on immune cells, for example, IL-1β secretion by human monocytes and macrophages [91], which attributes to many chronic inflammatory disease states [92]. In contrast, stimulation of β-adrenoreceptors has suppressive actions. Stimulation of β2-adrenergic receptors (β2AR) on DC and macrophages upregulates cyclic AMP (cAMP), activates protein kinase A, and inhibits the transcription factor NF-κB, thereby affecting cytokine production. For example, production of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-12, is downregulated, while production of the anti-inflammatory cytokine IL-10 is upregulated [93, 94]. These events result in the suppression of Th1 responses. In addition, adrenalin and noradrenalin influence other innate immune cells, such as NK cells, by reducing NK cell activity directly as well as indirectly through the inhibition of IL-12 and IFN-γ. Moreover, adrenalin and noradrenalin suppress the migration, phagocytosis, and degranulation of neutrophils [95].

Several studies have indicated the involvement of catecholamines in the pathogenesis of MS, as demonstrated by increased β-adrenergic receptor density on peripheral blood mononuclear cells (PBMC) from RR-MS patients [96] and discrepant noradrenalin and adrenalin levels in the PBMC from MS patients [97]. In addition, experimental studies in EAE have shown that selective depletion of noradrenalin levels in the CNS resulted in exacerbated clinical scores. Selective increase of CNS noradrenalin levels reduced astrocyte activation in the molecular layer of the cerebellum without affecting splenic Th1 or Th17 immune responses, thereby possibly providing benefit in EAE without affecting peripheral immunity.

Dopamine, another catecholaminergic neurotransmitter, also has important functions in the peripheral nervous system, as indicated by its release from peripheral nerve endings innervating lymphoid organs as well as from immune cells. Dopamine receptors are classified into two subgroups, dopamine-1 (D1)-like receptors (D1R and D5R) and D2-like receptors (D2R, D3R and D4R) [98]. In general, D1- and D2-like receptors are coupled to stimulation and inhibition of intracellular cAMP production, respectively [99]. In doing so, D1-like receptor-mediated increase of intracellular cAMP impairs the function of cytotoxic T lymphocytes (CTL) and regulatory T cells (Treg) [100, 101]. In contrast, it was reported that stimulation of D1-like receptors is involved in the polarization of naïve $CD4^+$ T cells towards Th17 cells [102]. D2-like receptor-mediated modulation of T cell function is demonstrated by IL-10-dependent induction of Treg [103], secretion of TNF-α from T cells indicative of a Th1 effector phenotype [103], and the differentiation of naïve $CD8^+$ T cells into CTL [103] as well as the modulation of the homing of T cells [104].

Similar to noradrenalin, dopamine levels are decreased in autoimmunity [105], suggestive of a protective role in the regulation of MS. Indeed, administration of a D2-like receptor agonist attenuates both the acute and the late phase of EAE [106], while administration of D2-like receptor antagonists worsened EAE pathology [102]. On the other hand, administration of D1-like receptor antagonists ameliorated EAE, which was associated with reduced IL-17 and increased IFN-γ levels. This finding was supported by previous results suggesting that dopamine signaling via D1-like receptors aggravates Th17-mediated diseases, such as MS, by promoting the IL-6/Th17 axis in conjunction with the suppression of Treg. Altogether, it is likely that D1-like receptors expressed on T cells are involved in the interface between autoimmunity and health. Indeed, decreased levels of D5R mRNA and protein have been found in PBMC from MS patients as compared to controls [107]. Noteworthy, dopamine reduced MMP-9 mRNA in controls and in IFN-β-treated MS patients, but not in untreated MS patients [107].

(2) *Parasympathetic Nervous System.* Acetylcholine (ACh) is the primary neurotransmitter of the parasympathetic nervous system (PNS). The PNS modulates immune responses through the efferent and afferent fibers of the vagus nerve. Two mechanisms demonstrating the inhibitory activity of the PNS on innate immune cells have been described [108].

First, direct stimulation of paraganglia cells by inflammatory cytokines, such as IL-1, results in signaling through afferent fibers. This leads to activation of parasympathetic brainstem regions to release ACh from efferent vagus nerves, thereby controlling inflammation through negative feedback. Subsequent binding of ACh to nicotinic receptors blocks the NF-κB signaling pathway. For example, stimulation of the α7-nAChR on macrophages, lymphocytes, and neutrophils inhibits NF-κB transcriptional activity and the production of inflammatory cytokines [109]. In addition, α4β2-nAChR activation modulates endocytosis and phagocytosis by macrophages [110]. Alternatively, ACh binds to the muscarinic ACh receptors (mAChaR). The M3 mAChR is expressed on T cells and has a role in the regulation of adaptive immune responses. Upon T cell receptor (TCR) stimulation, T cells release ACh, which stimulates M3 mAChR in an autocrine manner, thereby potentiating T cell activation and favoring differentiation towards a Th1 phenotype [111]. Hence, it can be summarized that the immunosuppressive or immunostimulatory consequences of ACh are dependent on the receptor type involved.

The second mechanism is indirect. When the peripheral cytokine-mediated inflammatory reaction stimulates the afferent sensory vagal route, a reflex response through the HPA axis that releases ACTH and GC is activated, which in turn reduces the production of pro-inflammatory cytokines.

A major region of cholinergic input, which plays an important role in learning and memory function, consists in the basal forebrain in the hippocampus [112]. Since the hippocampus is severely affected in MS patients as aforementioned [39], a selective imbalance in the hippocampal cholinergic neurotransmission exists in MS patients [113]. Accordingly, reduced synthesis of ACh is observed, possibly contributing to memory complaints as experienced by a significant proportion of MS patients [114].

3.3.2. Local Regulation by Neurotransmitters. Local regulation of the immune system is mediated by neurotransmitters which are synthesized in neurons and act on the postsynaptic neurons and other organs. Neurotransmitters are released from both the CNS and the peripheral nervous system as well as from immune cells including T cells, B cells, macrophages, DC, and granulocytes [4] thereby underscoring their possible contribution to the modulation of immune responses.

(1) *Glutamate.* Glutamate is a primary excitatory neurotransmitter in the CNS and has direct impact on neuronal activity [115]. Glutamate binds to ionotropic glutamate receptors (iGluR) or to metabotropic glutamate receptors (mGluR). Some G protein-coupled mGluR were recently reported to be involved in immune responses. For example, the expression of mGlu1R is induced after T cell activation and its ligation enhances the secretion of IL-2, IL-6, IL-10, TNF-α, and IFN-γ. In contrast, stimulation of mGlu5R, which is constitutively expressed on T cells, inhibits T cell proliferation through suppression of IL-6 production [116]. Hence, mGlu1R signaling counteracts the mGlu5R-mediated inhibitory effect on T cell proliferation.

Recent studies have identified glutamate as an important determinant of neurodegenerative damage in the course of MS [117]. It was shown that MS patients have increased glutamate levels in the brain [118] and in the CSF [119]. Furthermore, expression of iGluR and transporters is disturbed in MS [120] and in EAE [121]. Loss of glutamate transporters in cortical lesions correlates with microglial activation and synaptic damage [122]. In addition, overactivation of iGluR causes MS-like lesions [123], whereas iGluR antagonists exert beneficial effects in MS [124] and EAE by limiting oligodendrocyte and neuronal damage [125]. This increase in glutaminergic transmission observed in MS patients leads to excitotoxicity and neurodegeneration, resulting in cognitive impairments during the early phase of MS pathogenesis before the appearance of severe motor impairments. However, these actions may also be a consequence of a simultaneous dysfunction of GABA transmission, causing an imbalance between synaptic excitation and inhibition. Indeed, increased glutamate-mediated transmission and loss of GABAergic inputs were observed in EAE [126].

Besides, mGluR are also likely to contribute to glutamate transmission changes in MS and EAE. Indeed, it has been reported that mGlu1R expression in the cerebellum of MS patients and of mice with EAE is lower in comparison with controls, while the expression of mGlu5R is increased [127]. However, active MS lesions are characterized by increased expression of both receptors as well as the expression of mGlu2, -3, -4, and -8 [120]. Paradoxically, experimental studies in EAE have shown protective effects of these receptors. Indeed, treatment with a mGlu1R-selective enhancer resulted in ameliorated motor performance in EAE [127]. In addition, mGlu4R-deficient mice were more prone to develop EAE, which was associated with higher Th1/Th17 responses and increased production of inflammatory cytokines, such as IL-6, IL-12, and IL-23 [127]. Moreover, administration of a mGlu4R-selective enhancer increased resistance to EAE by inducing Treg, supporting the immunosuppressive effect of mGlu4R-mediated signaling [128].

(2) *Tachykinins.* Substance P and neurokinin A are closely related neurotransmitters and are both encoded by the same *Tac1* gene. Substance P is produced by the CNS and the peripheral nervous system, as well as by immune cells including monocytes, DC, and lymphocytes. It is a pro-inflammatory modulator of the immune response acting in either autocrine or paracrine fashion via the neurokinin (NK)-1 receptor, which is the primary receptor for substance P. Via activation of NF-κB in monocytes, substance P mediates increased production of pro-inflammatory mediators, such as IL-1β, IL-6, TNF-α, macrophage inflammatory protein (MIP)-1β, and IFN-γ [129]. In doing so, T cell proliferation as well as the generation of Th1 and Th17 cells is induced [130]. It was also shown that substance P regulates antigen presentation of DC [131], increases NK cell activity, and induces the release of CXCL8 and CCL2 from leukocytes as well as of vasoactive mediators, such as serotonin and histamine, from mast cells [132]. In MS plaques, substance P production has been demonstrated in activated macrophages [133] and astrocytes [134]. Although this may indicate a possible role for substance P in MS, no difference in substance P levels in the CSF from MS patients could be demonstrated as compared to healthy controls [135]. Whereas substance P directly acts on endothelial cells, resulting in increased vascular permeability [134] and subsequent enhanced permeability of the BBB, no interference with the induction of EAE in *NK-1*$^{-/-}$ mice could be observed [136]. Conversely, less severe clinical symptoms and reduced inflammation in the receptor-deficient mice were apparent which may indicate that substance P contributes to the maintenance of CNS inflammation during the chronic phase of EAE [136].

The NK-2 receptor exhibits the highest affinity for neurokinin A. Neurokinin A is known to control various vital responses in humans, such as airway contraction, vasodilatation, and vascular permeability [137]. The function of neurokinin A in the immune system is less well defined compared with the role of substance P. One study reported that neurokinin A stimulation induced mRNA expression of type I interferons, upregulated expression of MHC class II molecules, and antigen presentation by DC, thereby enhancing DC function [138] and subsequently inducing $CD4^+$ and $CD8^+$ T cell responses. Although this suggests involvement of NK-2 receptor-mediated signaling in chronic inflammation by excessive Th1-mediated immunity [138], no data describing a contributing factor of neurokinin A to the development or sustainment of MS have been reported.

(3) *Serotonin.* The neurotransmitter serotonin, also known as 5-hydroxytryptamine (5-HT), is produced by the CNS and regulates cognitive and endocrine functions, stress reactivity, circadian rhythm, and sleep [139]. Outside the CNS, serotonin is present in platelets, lymphocytes, monocytes, macrophages, mast cells, pulmonary neuroendocrine cells, enterochromaffin cells of the gut, and in some other cell types. Currently, at least 14 genetically, pharmacologically, and functionally distinct serotonin receptors have been identified. Among these, the serotonin-1A and serotonin-2A subtypes are of particular interest since they play a crucial role in the regulation of serotonergic neurotransmission and emotional and behavioral processes as well as the pathophysiology of various neuropsychiatric disorders [140]. These receptors are also expressed on immune cells and receptor activation appears to be both immunostimulatory and suppressive [141]. For example, through binding of the serotonin-1A receptor on monocytes, serotonin abrogates the monocyte-mediated suppression of NK cell functions [142], such as NK cell cytotoxicity, IFN-γ production by NK cells, NK cell proliferation, and expression of the CD16/56 NK cell antigen [142]. In contrast, serotonin decreases cAMP levels via the serotonin-1A receptor, which leads to stimulation of T cell proliferation [143], while ligation of the serotonin-2A receptor resulted in reduced lymphocyte proliferation [144] as well as decreased numbers of CTL [145].

Initial evidence for involvement of serotonin in autoimmunity comes from the experimental autoimmune neuritis (EAN) model. It was shown that blockade of the serotonin transporter by a selective serotonin reuptake inhibitor, thereby increasing the extracellular levels of serotonin, suppressed EAN [146]. Similarly, blockade of serotonin receptors also suppressed the development of EAE [147]. Furthermore, mice deficient for the serotonin transporter showed a milder disease course of EAE as compared to wild-type controls [148]. This was possibly mediated by a serotonin-dependent reduction of the inflammatory infiltrate in the CNS and by a reduction of the neuroantigen-specific production of IFN-γ by splenocytes. In addition, during the early paralytic stages of EAE, damage to the bulbospinal serotonergic neurons occurs, whereas neurologic recovery is associated with reestablishment of spinal serotonergic transmission. Damage to the bulbospinal serotonergic fibers also occurs in MS patients. This is reflected by reduced levels of 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of serotonin, in the CSF. Therefore, it is conceivable that degeneration of bulbospinal serotonin axons contributes to various neurologic manifestations of MS including autonomic and sensory symptoms [149].

(4) *Histamine.* Histamine is produced by histaminergic neurons located in the hypothalamus or released by mast cells, basophils, platelets, and enterochromaffin-like cells. Its major effects are related to sleeping, locomotor activity, exploratory behavior, food intake, awakening, and aggressive behavior [150]. Histamine can either inhibit or stimulate inflammatory reactions, depending on the type of receptor stimulated [151].

Upon histamine 1 receptor (H1R) ligation, histamine induces an increment of the secretion of the pro-inflammatory cytokines IL-1β, IL-6, and IL-8 and the chemokine CCL5 by peripheral macrophages [152]. Similarly, stimulation of H4R expressed on hematopoietic and immunocompetent cells involved in inflammatory responses also results in increased secretion of pro-inflammatory cytokines [153]. In addition, *in vitro* experiments indicated that histamine promotes Th1 responses through H1R and downregulates both Th1 and Th2 responses through H2R [154]. H1R and H4R ligation on $CD4^+$ T cells induces chemotaxis *in vitro*, whereas H1R and H2R modulate cytokine production. Another study indicated that binding of histamine to the H2R expressed on monocytes reduced the release of the pro-inflammatory cytokines IL-12 and TNF-α, while production of the anti-inflammatory cytokine IL-10 and Th2-like activity was increased [155]. Interestingly, expression of different histamine receptors is differentially regulated, depending on the stage of differentiation and of activation of target cells thereby potentially explaining variation in experimental data from diverse studies [156].

Already in 1983, it was noted that histamine may be involved in MS, as evidenced by 60% higher histamine levels observed in MS patients as compared to healthy controls [157]. Since then, several experimental studies confirmed the role of histamine in MS. Upregulated expression of H1R was shown in MS lesions [158], whereas epidemiological studies demonstrated a protective effect of H1R antagonists capable to cross the BBB in MS [159]. Further evidence was provided by a study showing the requirement for *Hrh1* gene expression for susceptibility to EAE [160]. Indeed, H1R-deficient mice exhibit a significant delay in the onset of EAE and a reduction in the severity of clinical signs compared with wild-type mice [160]. In addition to H1R, H2R also seems to partially regulate encephalitogenic Th1 responses and EAE susceptibility. Indeed, $H2R^{-/-}$ mice develop less severe disease than wild-type mice during the acute and early phase [161], possibly mediated by H2R-dependent abrogation of pro-inflammatory cytokine production.

Although H1R and H2R have a clear pro-inflammatory role and disease-promoting effect, H1R and H2R activation may also play an important role in limiting autoimmune responses [162]. It was shown that histamine ligation of H1R and H2R inhibits the proliferation of murine $CD3^+$ T cells directed against myelin-derived antigens *in vitro*, as well as their adhesiveness to the inflamed endothelium [163]. Accordingly, treatment with an H2R agonist reduces the clinical signs in EAE [164]. Furthermore, $H4R^{-/-}$ mice develop more severe EAE, accompanied by increased neuroinflammatory signs and increased BBB permeability, with a higher proportion of infiltrating Th17 cells than Treg, as compared to wild-type mice [165].

(5) *Gamma-Aminobutyric Acid.* γ-Aminobutyric acid (GABA) is the most prominent inhibitory neurotransmitter in the CNS [166]. In the immune system, GABA receptors are expressed on lymphocytes [167] and peripheral macrophages [168]. GABA has similar anti-inflammatory actions as GC.

Indeed, GABA negatively modulates the levels of pro-inflammatory cytokines produced by macrophages [169] as well as cell proliferation [170] and migration [171].

Loss of GABAergic innervations is a physiologic hallmark of MS and EAE. Additionally, it was shown that GABA is decreased in the serum and CSF of MS patients and in EAE [172, 173]. Reduced GABA-related gene transcripts and density of inhibitory interneuron processes in motor cortex samples from MS patients were also reported [174] as well as irreversible alterations of GABA transmission in the striatum of EAE mice. Increasing GABA concentration in the CNS delayed EAE onset and reduced severity of symptoms following EAE induction. In mice with established EAE, it reversed paralysis and decreased the number of relapses [175]. Moreover, the chronic persistence of pro-inflammatory cytokines in EAE induced profound alterations in the electrophysiological network properties in cultured cortical neurons, which were reverted by GABA administration [176]. This was further supported by demonstrating inhibition of GABA transmission in mouse brain slices upon administration of CSF from MS patients with MRI-confirmed active brain lesions. The investigators concluded that focal inflammation in MS perturbs the cytokine milieu within the CSF, resulting in diffuse GABAergic alteration in neurons [177].

4. Regulation of the Neuroendocrine System by the Immune System and Dysfunction in MS

Given the bidirectional interactions of the neuroendocrine and the immune systems, the immune system also regulates the neuroendocrine system through the secretion of cytokines. Cytokines are immune mediators produced in response to antigens and toxins or after stimulation by other cytokines. Cytokines and their receptors are expressed in the neuroendocrine system and exert their effects both centrally and peripherally [178, 179]. Inflammation in the CNS contributes to the onset and progress of neurodegenerative diseases, including MS [180]. Indeed, pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α play an important role during the pathophysiological processes involved in the disease pathogenesis and course of MS. Through several mechanisms, including humoral, neural, and cellular pathways, cytokines are able to reach the brain. On the one hand, they can enter the brain through the areas with a poorly developed BBB or via active transport across the BBB. On the other hand, these cytokines can be expressed and released from resident cells in the CNS, including glial cells, neurons, endothelial cells, or invading immune cells [181]. Moreover, cytokines that are produced in the periphery activate primary afferent nerves, such as the vagus nerves. In doing so, cytokines stimulate neurons to modulate the social interaction [182], the stressful HPA axis responses [183], and the activities of the autonomic nervous system [184].

Excessive pro-inflammatory cytokine production is physiologically joined to a simultaneous increment of the synthesis of anti-inflammatory cytokines, inhibitory neurotransmitters, and GC. The resulting equilibrium is called homeostasis. However, prolonged increased HPA axis activity results in a prompt loss of the anti-inflammatory mediators with an increase of pro-inflammatory mediators [185], thereby ultimately contributing to a state of disease. An altered cytokine balance has been observed in MS patients, as evidenced by increased pro-inflammatory cytokine levels in the periphery and in the CNS. Indeed, elevated mRNA and protein levels of IL-1β, IL-6, and TNF-α have been reported in CNS lesions, CSF, and peripheral blood monocytes of MS patients [186, 187] as well as in EAE [188]. Additionally, activated astrocytes and microglial cells express a large number of cytokines and chemokines which subsequently contribute to neuroinflammation in MS. These brain-derived cytokines also act to protect from or enhance neuronal cell death. In doing so, cytokine-mediated neuronal cell death is considered to be important in several neurodegenerative diseases, such as MS.

4.1. Cytokine-Mediated Regulation of Hormones. Whereas interferons were the first cytokines shown to exert neuroendocrine effects as demonstrated by increased steroid production upon interferon treatment, it is now clear that several cytokines have functions in the neuroendocrine system. Indeed, IL-1, IL-2, IL-6, IL-10, IFN-β, IFN-γ, leukaemia inhibitory factor (LIF), TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) can stimulate the HPA axis to release GC. In particular, these cytokines have been reported to elevate plasma GC levels in both humans and animal models [189–193] via stimulation of CRH and ACTH production in hypothalamic and pituitary tissues, respectively. In addition, melatonin release by the pineal gland is stimulated by IFN-γ, granulocyte colony-stimulating factor (G-CSF), and GM-CSF [194, 195]. In contrast to the HPA axis, inflammatory cytokines have negative effects on the HPG axis, resulting in reduced gonadal functions [196].

4.2. Cytokine-Mediated Regulation of Neurotransmitters. Activation of innate immune responses, by pathogens as well as by damage-associated molecules, leads to the release of inflammatory cytokines that signal the CNS via the subdiaphragmatic vagus nerve, thereby resulting in changes that are associated with sickness behavior, such as fever [197]. Pro-inflammatory cytokines, such as IL-1, IL-2, IL-6, IFN-β, IFN-γ, LIF, and TNF-α, stimulate the SNS to release noradrenalin. Furthermore, IL-1β enhances the inhibitory effects of GABA. Given the inhibitory effect of these neurotransmitters on inflammation, this negative feedback loop will stop inflammation. In addition, IL-1β administered systemically or in the brain resulted in subsequent increased extracellular levels of serotonin in the anterior hypothalamus and in the hippocampus [198].

Several cytokines are also involved in the regulation of sleep and wakefulness [199], including IL-1β, IL-1 receptor antagonist, IL-2, IL-2 receptor, IL-4, IL-6, IL-9, IL-10, IL-13, IL-18, TGF-β, IFN-α, IFN-γ, TNF-α, and TNF-α receptors p55 and p75 [200–203]. Pro-inflammatory cytokines are more likely to induce sleep, whereas anti-inflammatory

cytokines show antisomnogenic effects or do not influence sleep-wake regulation.

Chemokines, a large group of proteins from the cytokine family that are pivotal in leukocyte migration, were found to play a role in signaling functions in the CNS [204]. Macrophages, glial cells, and also neurons are able to constitutively express chemokines and multiple chemokine receptors, which may function as neuromodulators in the homeostatic brain. In neurons, chemokines are located in central nerve endings in small clear and dense core vesicles [205], where they colocalize with traditional neurotransmitters and are released following membrane depolarization [206]. CXCL12 and its receptor CXCR4 [207, 208] as well as CCL2 and its receptor CCR2 are constitutively expressed by mesencephalic dopaminergic neurons [209]. Therefore, both chemokines can modulate the electrical activity of dopaminergic neurons. Furthermore, CCL2 can be upregulated by cells surrounding the sites of brain injury and can attract progenitor cells for healing purposes [210].

The aforementioned hormones, neurotransmitters, and cytokines with their immunomodulatory activity are summarized in Table 1. A comprehensive overview of the interaction between the neuroendocrine system and the immune system is depicted in Figure 1.

5. Intervening with the Neuroendocrine Immune System for Treatment of MS

To date, none of the available therapies for MS are curative. Their primary aims are inducing remission after relapse, reducing the number of new relapses, and preventing or slowing the progression of disability. During acute relapse, patients may be hospitalized and symptomatically treated with high doses of corticosteroids. Additionally, a number of disease-modifying treatments have been approved, albeit mostly only for RR-MS. These include IFN-β, glatiramer acetate, natalizumab and fingolimod. Whereas their relative success in RR-MS patients supports the role of the immune system in demyelination and axonal loss, these drugs are not sufficient to stop accumulation of disability. Management of these deficits is therefore also important [211]. Here, we will focus on treatment modalities that primarily intervene at the level of the neuroendocrine system.

5.1. Management of Relapse Using Glucocorticoids. Since the 1950s, GC are widely used for the suppression of inflammation in chronic inflammatory diseases such as asthma, RA, MS, and other autoimmune diseases. Despite the introduction of disease-modifying therapies, GC therapy remains the first-line treatment upon relapse for inducing remission in MS sooner and with fewer deficits for the patient. Methylprednisolone is among the most commonly used corticosteroids in MS and reduces the number of gadolinium-enhancing lesions during MS exacerbations [212]. This effect is mediated by dampening the inflammatory cascade, inhibiting the activation of T cells, and decreasing migration of immune cells into the CNS [213]. The optimal dose, frequency and duration of treatment, and route of administration of methylprednisolone are constantly being investigated for improvement of patient care. One study reported that high doses of methylprednisolone were more effective for treatment of relapses, whereas low doses of methylprednisolone correlated with disease reactivation [214]. Studies suggest that GC administered orally are equally effective at treating MS symptoms as intravenous treatment [215, 216]. To date, little is known about the effect of long-term treatment on disease progression in patients with MS [217]. Nevertheless, Then Bergh et al. have reported reduction of inflammatory disease activity and T2 lesion volume in RR-MS by a single monthly methylprednisolone infusion without clinically relevant side effects [218]. Furthermore, different combination treatment regimens are under evaluation in order to achieve synergism and improve MS management [219–221].

Although the majority of patients with MS benefits from GC treatment, a small set of patients fails to adequately respond, suggesting differences in sensitivity to GC, a phenomenon recognized as GC resistance [222]. Given the important role of endogenous GC in controlling the immune system, GC resistance may be associated with the disease course or the susceptibility of MS. However, conflicting results are reported by studies investigating *in vitro* GC resistance in MS. Whereas some have demonstrated reduced sensitivity of patients' white blood cells to GC treatment in order to suppress lymphocyte function [223], others have found no differences [224] as compared to healthy controls. Observations of reduced GC sensitivity have been made in other autoimmune diseases or inflammatory diseases, including RA and asthma [225, 226], and several factors have been identified contributing to GC resistance, such as reduced GR expression [227]. Although the mechanisms for GC resistance in MS remain to be further explored, these results may suggest implications for treatment efficacy, at least in a subgroup of MS patients.

Because of the aforementioned effects of circadian rhythms on the symptoms of autoimmune and inflammatory diseases, there is a growing interest in the efficacy of timed treatment or so-called chronotherapy. Although the impact of chronotherapeutics on treatment success remains to be fully elucidated, beneficial effects of chronotherapeutics have been identified in the management of MS and RA [228, 229] as evidenced by significantly improved clinical recovery upon nighttime treatment with GC [230].

5.2. Lifestyle Interventions and Physical Rehabilitation. Different lifestyle interventions can influence the neuroendocrine-immune system, including physical exercise. Physical exercise triggers a systematic series of neuroendocrine and immune events directed at accommodating the human body to the increase in physiological demands. Furthermore, the neuroendocrine-immune system can adapt to chronic overload or exercise training. Because of the vital role of the neuroendocrine system at maintaining homeostatic control during exercise, one exercise bout results in an increase of hormonal levels, including growth hormone, testosterone, cortisol, ACTH, adrenalin, noradrenalin, and estradiol [243].

Table 1: Neuroendocrine factors and their immunomodulatory effects.

Substance	Receptor	Effect on immune response	Reference
Acetylcholine	Muscarinic acetylcholine receptor (mAChR)	Differentiating towards a Th1 phenotype	[111]
	Nicotinic acetylcholine receptor (nAChR)	Inhibits IL-1β, IL-6, IL-18, and TNF-α production	[109]
ACTH	ACTH receptor	Inhibits IFN-γ production and Ig production and blocks macrophage activation by IFN-γ	[231]
Adrenalin/ noradrenalin	α-Adrenergic receptors	Upregulation of cAMP; inhibits IL-1, IL-6, IL-12, and TNF-α production; enhances IL-10 production	[91]
	β-Adrenergic receptors	Downregulation of cAMP	[93, 94]
Cortisol	Glucocorticoid receptor (GR)	Inhibits IFN-γ, IL-2, IL-6, and TNF-α Enhances IL-4 and TGF-β production Enhances immune cell expression of IL-1, IL-2, IL-6, and IFN-γ receptors	[10, 232, 233]
CRH	Corticotropin-releasing hormone receptor	Activates macrophages Inhibits IL-1 and IL-6 production	[231]
Dopamine	D1-like receptors	Upregulation of cAMP	[99]
	D2-like receptors	Downregulation of cAMP	[99]
GABA	GABA receptors	Reduces the proliferative response of activated $CD8^+$ T cells Reduces IL-6 release	[169, 170]
Glutamate	mGluR1	Enhances IL-2, IL-6, IL-10, TNF-α, and IFN-γ production	[116]
	mGluR5	Inhibits IL-6 production	[116]
Growth hormone	Growth hormone receptor	Activates macrophages and enhances H_2O_2 production	[234]
Gonadotropin-releasing hormone	Gonadotropin-releasing hormone receptor	Increases IL2R expression, T- and B-cell proliferation, and serum Ig	[235, 236]
Histamine	Histamine 1 receptor, histamine 4 receptor	Enhances IL-1β, IL-6, IL-8, and RANTES production Induce chemotaxis of $CD4^+$ T cells	[152–154]
	Histamine 2 receptor	Inhibits IL-12, IFN-γ and TNF-α, and enhances IL-10 production	[155]
Luteinizing hormone	Luteinizing hormone/choriogonadotropin receptor	Enhances IL-2 stimulated T-cell proliferation	[237]
Melatonin	Melatonin receptor	Enhances IL-1, IL-2, IL-6, and IFN-γ production	[238, 239]
Neurokinin A	Neurokinin 2 receptor (NK2-receptor)	Enhances mRNA expression of IFN-α and IFN-β Enhances DC function	[138]
Estrogen	Estrogen receptor	Enhances T-cell proliferation and activity IFN-γ gene promotor	[240]
Progesterone	Progesterone receptor	Enhances IL-4 production and CD30 expression	[85, 86]
Prolactin	Prolactin receptor	Enhances T cell proliferation, IFN-γ, IL-2 receptor expression, and macrophage function	[42, 43]
Serotonin	Serotonin-1a receptor	Enhances NK cell cytotoxicity Downregulation of cAMP Stimulation of T-cell proliferation	[142, 143]
	Serotonin-2a receptor	Inhibits lymphocyte proliferation	[144]
Substance P	Neurokinin 1 receptor (NK1-receptor)	Enhances IL-1β, IL-6, TNF-α, MIP-1β, and IFN-γ production Enhances T-cell proliferation Enhances NK cell cytotoxicity	[129, 130, 132]
Vasopressin	Vasopressin receptor	Enhances IFN-γ production	[241]
VIP	Vasoactive intestinal peptide receptor	Inhibits T-cell proliferation and IL-12 Enhances IL-5 and cAMP production	[242]

On the other hand, the immune system is also important in maintaining homeostatic control during and after physical exercise. Changes that occur following an exercise bout include altered counts of peripheral blood leukocytes [244] as demonstrated by increased concentrations of neutrophils and lymphocytes [245] as well as increased serum concentrations of pro- and anti-inflammatory cytokines and acute phase proteins [246]. Furthermore, long-term exercise training has been shown to reduce basal cytokine levels and low-grade inflammation [247]. However, this could not be reproduced by others who reported no effect of long-term exercise on basal cytokine levels, albeit that a decrease of C-reactive protein (CRP) levels was observed [248].

Aforementioned observations triggered the interest to use physical exercise in MS patients in order to manage disease-related impairments. It was shown that physical exercise beneficially affects quality of life, symptoms including depression, fatigue, and possibly cognitive functions in MS patients [249]. Since it is becoming increasingly clear that these neuropsychiatric symptoms of MS are, at least in part, mediated by biological processes such as inflammation, neuroendocrine dysfunction, or regional brain damage, physical exercise may successfully affect the underlying biology and slow down the disease process [250]. Besides, several studies evaluated the effect of physical exercise on disease progression in MS patients using the expanded disability status scale (EDSS) score. In general, these studies did not found any change after either endurance training [251–253], resistance training [254–256], or combined training interventions [257, 258]. In contrast, one study reported an improvement in EDSS score upon a combined training program [259]. Alternatively, a protective effect of cardiorespiratory fitness on brain function and structure in MS patients has been demonstrated using MRI [260, 261].

To date, the mechanisms linking physical exercise and disease status in MS patients remain, however, to be elucidated [262]. It is possible that physical exercise counteracts imbalances between pro-inflammatory Th1 cytokines and anti-inflammatory Th2 cytokines [263]. A few studies have addressed the effect of physical exercise on cytokine levels in MS patients, although conflicting results were reported. On the one hand, IL-4, IL-10, CRP, and IFN-γ levels were reduced in MS patients after 8 weeks of biweekly resistance training [264]. Similarly, it was shown that IL-17 and IFN-γ levels were reduced in MS patients after 8 weeks of combined endurance and resistance training [259]. In contrast, elevated IFN-γ and TNF-α levels in MS patients after 8 weeks of endurance training were demonstrated, whereas no changes were observed in healthy controls [265]. These effects of physical training on the immune system may indirectly be mediated via modulation of the neuroendocrine system. Indeed, White et al. showed increased β1 and β2 adrenergic receptor expression in MS patients upon a moderate exercise bout as compared to controls [266].

5.3. Clinical Testing of New Treatment Modalities

5.3.1. Estrogen. Several studies in EAE have shown the inhibitory effects of estrogens on disease pathogenesis [191, 267, 268]. Indeed, estrogen treatment before induction of EAE delays onset of disease and reduces disease activity. Protective mechanisms of estrogen treatment in EAE involve anti-inflammatory processes including decreased production of pro-inflammatory cytokines, such as TNF-α, and induction of Treg. Furthermore, decreased expression of MMP-9 by T cells was reported, resulting in reduced infiltration of T cells into the CNS [58]. Based on these findings, several clinical trials investigating estrogen administration in MS are underway [269, 270].

In a first pilot crossover trial, 6 female RR-MS patients were treated with 8 mg estriol per day during 6 months, followed by a 6-month posttreatment period and a subsequent retreatment period during 4 months. The investigators reported reduced number and volume of gadolinium-enhancing lesions upon estriol treatment [269]. A multicenter randomized double-blind placebo-controlled phase II trial was recently started at the University of California in order to investigate the therapeutic effect of oral estriol treatment in combination with glatiramer acetate treatment in female RR-MS patients (http://www.clinicaltrials.gov/ct2/show/NCT00451204). The European POPART'MUS study, an ongoing double-blind placebo-controlled phase III trial, designed for women with MS in their postpartum period, aims at the reduction of postpartum relapses by administration of estradiol and progestin. High doses of progestin in combination with endometrial-protective doses of estradiol will be given immediately after delivery and continuously during the first three months postpartum [270]. Although the first results of therapeutic use of estrogen in MS are encouraging, more research is warranted in order to understand the estrogen-mediated underlying mechanisms. The outcomes of the currently ongoing MS trials may help to clarify therapeutic use of estrogen in combination with first-line immunomodulatory drugs.

For completeness, also the effect of testosterone was evaluated in a first pilot study including 10 men with RR-MS. A daily treatment with 10 g of a 100 mg testosterone-containing gel for 12 months resulted in improvement of cognitive performance and delayed progression of brain atrophy. These findings suggest that testosterone treatment is safe and well-tolerated and may have neuroprotective effects in men with RR-MS [271].

5.3.2. Neurotransmitters

(1) *Catecholamines.* By increasing noradrenalin levels through administration of tri- and tetracyclic antidepressants and L-dopa, the course of MS was ameliorated [272]. Indeed, after 1-2 months of treatment approximately 75% of patients experienced substantial improvements in sensory, motor, and autonomic symptoms. Moreover, these patients regained functions that were lost for several years. Interestingly, also treatment with IFN-β, which is a widely used and approved immunomodulatory therapy for MS, was shown to substantially elevate the catecholamine levels in PBMC of MS patients

[273]. This suggests that the improvement in MS during IFN-β treatment is, at least in part, mediated by increased levels of catecholamines.

(2) *Acetylcholine.* Based on experimental evidence that ACh promotes production of anti-inflammatory cytokines [109], it was demonstrated that a cholinesterase inhibitor can alleviate neuroinflammatory responses in the EAE model thereby reducing clinical and pathologic severity of EAE [274]. In several phase I/II clinical studies using cholinesterase inhibitor therapy, beneficial effects on cognitive deficits in MS were observed [275, 276]. Indeed, following treatment with rivastigmine, a widely used ACh esterase inhibitor for the treatment of Alzheimer's disease, Shaygannejad et al. reported a modest, but significant improvement of memory in MS patients with Wechsler Memory Scales (WMS) confirmed mild verbal memory impairment [276]. Nevertheless, similar improvements were observed in placebo-treated MS patients. Additionally, treatment of MS patients with donepezil, an alternative ACh esterase inhibitor, showed significant improvement in memory performance on the selective reminding test, a test of verbal learning and memory, as compared to placebo-treated MS patients. Moreover, cognitive improvement was reported by clinicians in twice as many donepezil versus placebo-treated MS patients. In addition, the donepezil-treated MS patients themselves reported more often memory improvement than placebo-treated MS patients [275].

(3) *Glutamate.* Since extracellular accumulation of glutamate contributes to excitotoxic injury of neurons and glial cells, inhibition of glutamate might be beneficial in MS patients. For this, Killestein et al. examined the effect of one year riluzole treatment in MS patients [277]. Riluzole is a neuroprotective agent that inhibits the release of glutamate from nerve terminals. Moreover, it modulates iGluR and inhibits excitotoxic injury in several experimental models of neurodegenerative disease [278]. The investigators reported a reduction in the rate of brain and cervical cord atrophy as well as in the development of T1 hypointense lesions on MRI in primary progressive MS.

(4) *Serotonin.* Experimental evidence from animal studies has shown an immunosuppressive role of serotonin in autoimmunity. Sijens et al. evaluated the impact of elevated extracellular levels of serotonin mediated by fluoxetine, a selective serotonin reuptake inhibitor used as antidepressant, in MS patients [279]. By using diffusion tensor imaging (DTI) and ^{1}H magnetic resonance spectroscopy (MRS), the investigators reported partial normalization in diffusion and metabolic properties of brain tissue upon 2-week treatment with fluoxetine, thereby providing evidence for a possible neuroprotective effect of fluoxetine in MS.

(5) *Histamine.* Ligation of the histamine receptor, H1R, on immune cells induces secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, and the chemokine CCL5 [152]. Therefore, treatment with H1R antagonists would reduce the secretion of pro-inflammatory cytokines. Indeed, treatment of MS patients with hydroxyzine, a well-known H1R antagonist, stabilized or improved the neurological status of 75% of treated MS patients, as assessed by Kurtzkes's EDSS [280].

5.3.3. Cytokines. In 1993, interferon (IFN)-β was the first product to be approved by the FDA as disease-modifying treatment for MS. To date, these include three different commercial formulations which have been demonstrated to reduce the inflammatory process in MS by decreasing the secretion of pro-inflammatory cytokines, increasing anti-inflammatory cytokine levels, and reducing the number of immune cells migrating towards the CNS. In doing so, IFN-β decreases relapse rate, increases time between relapses, and decreases the severity of relapses, while decreasing the amount of accumulated lesions seen on MRI.

In addition, targeting cytokine production has been intensively investigated as a potential treatment strategy in autoimmunity [281]. One of the greatest successes in immunology is the treatment of RA with anti-TNF-α therapy. Unfortunately, TNF neutralization in MS patients exacerbated disease symptoms [282]. Similarly, treatment with tocilizumab and anakinra, humanized monoclonal antibodies competing for receptor binding with IL-6 and IL-1β, respectively, has been approved in RA. However, the safety and efficacy of anakinra, tocilizumab, or administration of other IL-1- and IL-6-targeting compounds have not yet been evaluated in MS patients [283]. Furthermore, also IL-12 and IL-23, interleukins sharing p40 as a common subunit, have a clear role in the pathogenesis of MS because of their respective function in Th1 and Th17 differentiation. Ustekinumab, a monoclonal antibody that neutralizes the p40 subunit, is effective in patients with psoriasis or psoriatic arthritis, and in patients with Crohn's disease. Unfortunately, ustekinumab failed to show any efficacy in RR-MS patients [284]. In summary, although targeting cytokines as therapy for MS is a feasible approach, careful consideration must be given to the highly pleiotrophic character of the cytokine as well as the stage of the disease process being targeted.

6. Conclusion

Although knowledge of the immunopathogenesis as well as genetic predisposition of MS has greatly increased over the last decades, potential environmental triggers such as stress and pregnancy may not be underestimated in order to better understand how these factors modulate disease. In this perspective, it is clear that the neuroendocrine-immune system has an important role in the pathogenesis of autoimmune diseases, including MS. Here we have provided an overview of the complex system of crosstalk between the neuroendocrine and immune system, whereby they share an extensive range of common messenger molecules and receptors and whereby they can monitor each other's activities. Discrepancies at any level can lead to changes in susceptibility to and to severity of several autoimmune and inflammatory diseases. These principles are now being used to test novel therapies for MS

based on addressing and correcting the dysregulation of these neural and neuroendocrine pathways.

However, the key question that remains unanswered is whether these alterations in neuroendocrine pathways and receptors are involved in the pathogenesis of MS as a predisposing factor or whether they are a result of the inflammatory status of the disease. Based on preliminary evidence that hormonal changes may appear before the symptomatic phase of the disease [285, 286], it is tempting to speculate that a pro-inflammatory hormone favors the rupture of tolerance, which is a key feature of autoimmunity.

In conclusion, dysfunction of the neuroendocrine-immune system in patients with autoimmune diseases, including MS, seems to be important in the pathogenesis of these diseases. Increasing the knowledge of the neuroendocrine-immune system in MS can help to elucidate the underlying mechanisms of the inflammatory responses in MS and mutatis mutandis in other autoimmune diseases. Furthermore, intensive research on the modulatory function of the neuroendocrine-immune system may provide new therapeutic approaches for the treatment of MS in the near future.

Authors' Contribution

Nathalie Deckx and Wai-Ping Lee contributed equally and are the co-first authors.

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Research Article

An Imbalance between Frequency of CD4+CD25+FOXP3+ Regulatory T Cells and CCR4+ and CCR9+ Circulating Helper T Cells Is Associated with Active Perennial Allergic Conjunctivitis

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Allergic conjunctivitis (AC) is one of the most common eye disorders in ophthalmology. In mice models, it has been suggested that control of allergic conjunctivitis is a delicate balance between Tregs and inflammatory migrating effector cells. Our aim was to evaluate the frequency of Tregs and the frequency of homing receptors expressing cells in peripheral blood mononuclear cells (PBMC) from patients with perennial allergic conjunctivitis (PAC). The analyses of phenotypic markers on CD4+ T cells and both soluble or intracellular cytokines were performed by flow cytometry. CD4+CD25+ cells were 15 times more frequent in PBMC from patients than HC; the vast majority of these CD4+CD25+ cells were FOXP3−, and most of CD4+ T cells were CCR4+ and CCR9+ cells. Upon allergen-stimulation, no significant changes were observed in frequency of Treg; however, an increased frequency of CD4+CCR4+CCR9+ cells, CD4+CD103+ cells and CD4+CD108+ cells with increased IL-5, IL-6, and IL-8 production was observed. These findings suggest an immune dysregulation in PAC, characterized by diminished frequency of Tregs and increased frequency of circulating activated CD4+ T cells; upon allergen-stimulation, these cells were expressing cell-surface molecules related to mucosa homing and were able to trigger an inflammatory microenvironment.

1. Introduction

Allergies represent the most frequent chronic diseases worldwide [1]; ocular allergy is one of the most common ocular conditions encountered in clinical practice. Allergic conjunctivitis (AC) includes a spectrum of a number of traditional overlapping conditions that range from intermittent to persistent signs and symptoms, and these are fluctuating in severity and presentation. AC could be as mild forms with transient inflammation, such as seasonal (SAC) and perennial allergic conjunctivitis (PAC), or as more severe persistent and chronic inflammatory forms such as vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) [2, 3]. Allergic conjunctivitis is initiated by the predominant activation of CD4+ T cells to environmental allergens, culminating in a Th2 response with generation of IgE antibodies [4]. The CD4+ T cells from allergic patients are resistant to apoptosis and produce large amounts of IL-5 [5], favouring chronicity and perpetuating inflammation and relapsing-remitting symptoms. It is well known that in the chronic forms of allergic conjunctivitis CD4+ T cells are able to migrate to the ocular mucosa, maintaining the inflammatory process [6]. In

mice models of ocular allergy, it has been demonstrated that CD4+CD25+FOXP3+ regulatory T cells (Tregs) influence the expression of immune-mediated allergic inflammation in conjunctiva, [7] counteracting inflammation through anti-inflammatory cytokines such as TGF-β and IL-10 [7, 8]. Therefore, it appears that control of allergic conjunctivitis is a delicate balance between Tregs and inflammatory migrating effector cells. In humans, intraepithelial leukocytes in the ocular surface express human mucosal lymphocyte antigen (HML or CD103) in nonpathological conditions [9], whereas in chronic allergic status, infiltrating CD4+ T cells are CCR3+ and/or CXCR3+ cells [10, 11] Nevertheless, the molecules involved in T-cell homing to conjunctiva, during the acute forms of AC in humans, are not fully studied yet. The aim of this study was to characterize the immunophenotypical features of circulating helper T cells, associated with Treg phenotype and homing receptors, in patients with perennial allergic conjunctivitis.

2. Methods

2.1. Patients. 21 individuals (12 males and 9 females, mean age 11.3 years, range 5–17) with active perennial allergic conjunctivitis (PAC) were studied. Perennial allergic conjunctivitis diagnosis was based on clinical history (mean disease duration 3.5 (SD 3.1) years) and eye and physical examination. Seven healthy volunteers were used as controls (4 males and 3 females, mean age 10.2 years, range 7–15). All participants gave their informed/assent consent for blood sampling after written information was provided. The study adhered to the ethical principles of the Declaration of Helsinki, the E11 Statements of International Conference of Harmonisation (E11-ICH), and was approved by the Institutional Ethics Committee Board at the Institute of Ophthalmology "Fundación Conde de Valenciana", Mexico City.

2.2. Monoclonal Antibodies and Reagents. Phycoerythrin (PE) labelled-mouse monoclonal antibodies (mAbs) against human CD25, CD103, CD108, IL-5; PECy5-labelled mAbs anti-human CD4; and fluorescein isothiocyanate (FITC)-labelled antibodies against human FOXP3 were purchased from BD PharMingen (San Jose, CA, USA). FITC-labelled mAbs anti-human CCR9 and CCR7 and PE-labelled mAbs anti-human CCR4 were from R&D Systems (Minneapolis, MN, USA). Allophycocyanin (APC)-labelled mAbs against CD4 were purchased from e-Biosciences (San Diego, CA, USA). Lymphoprep (Ficoll 1.077 density) was obtained from Nycomed Pharma (Nyegaard, Oslo, Norway). RPMI-1640 culture medium, Concanavalin A (Con A), PMA, ionomycin, saponin, brefeldin-A, and salts were from Sigma Chemical Co. (St. Louis, MO, USA). Sodium pyruvate, L-glutamine, and 2-mercaptoethanol were purchased from Gibco BRL (Rockville, MD, USA). Fetal calf serum was from HyClone Labs (Logan, UT, USA), *Dermatophagoides pteronyssinus (Der p)* was purchased from Allerstand Co. (Mexico, DF, MEX).

2.3. Peripheral Blood Mononuclear Cells. Whole heparinized peripheral blood was diluted 1 : 2 (vol/vol) in phosphate buffered saline (PBS), pH 7.2. Peripheral blood mononuclear cells (PBMC) were separated on a Ficoll density gradient by centrifugation at 1700 rpm for 30 min at room temperature. After centrifugation, the cells in the interface were collected, washed twice, and counted using a handheld automated cell counter (Millipore Co., Billerica, MA, USA), and viability was assessed by eosin dye exclusion.

2.4. Immunofluorescence Staining of Cell Surface Markers. Double or triple-colour staining was performed on PBMC by direct immunofluorescence, using APC- or PECy5-mAb anti-CD4 and either FITC- and/or PE-labelled mAbs against CD25, CD103, CD108, CCR4, CCR7, or CCR9. Briefly, 2×10^5 cells were suspended in 20 μL PBS supplemented with 0.2% bovine serum albumin and 0.2% sodium azide (PBA) and were incubated with fluorochrome-labelled mAb for 30 min at 4°C. After incubation, the cells were washed twice with PBA, fixed with 1% p-formaldehyde, and analysed by flow cytometry.

2.5. Immunofluorescence Staining of Intracellular Markers. Stimulated or nonstimulated PBMC were washed with PBA and stained with APC- or PECy5 labelled mAbs against CD4 and/or PE labelled mAbs against CD25 for 30 min. After washing, the cells were fixed with 4% p-formaldehyde in PBS for 10 min at 4°C. The cells were washed twice with PBS and permeabilised with saponin buffer (0.1% saponin and 10% BSA in PBS) by shaking gently for 10 min at room temperature. The cells were then incubated with FITC-labelled anti-human FOXP3 antibodies and/or PE-labelled anti-human IL-5. In all cases isotype-matched controls were used.

2.6. Cell Cultures. PBMC were cultured in 96-well flat bottomed cell culture plates (Costar, Cambridge, MA, USA) at 2×10^5 cells/well in RPMI-1640 medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/mL gentamicin, and 0.5% heat-inactivated fetal calf serum and incubated at 37°C in a 5% CO_2 humidified chamber. After 24 h the culture medium was removed, and fresh culture medium supplemented with 10% heat-inactivated fetal calf serum and *Der p* (7.5 μg/mL) were added. After 7 days of culture, the cells were harvested and processed to measure intracellular FOXP3 expression, and homing receptors on cell surface by flow cytometer. The Con A mitogen (2 μg/mL) was used as a cell stimulation positive control. Supernatants were collected and stored at −70°C to determine soluble cytokines. In order to determine intracellular IL-5, four hours before antigen or polyclonal cultures ended, brefeldin-A was added (10 μg/mL), and at the end of the incubation period the cells were harvested and were processed to immunofluorescence staining as described above.

2.7. Flow Cytometric Analysis. All cells were analysed for the expression of phenotypic markers on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using cell quest

software, and 10000 events were counted. To analyse the staining of cell-surface markers, the lymphocytes were first gated by their physical properties (forward and side scatter), then a second gate was drawn based on immunofluorescence characteristics of the gated cells, assessing fluorescence intensity by histograms. To determine Tregs the cells were gated on FSC-SSC dot plot, then the lymphocytes were gated on CD4+ T cells in a SSC-CD4 dot plot, then CD4+ cells were selected, and a CD4-CD25 dot plot was created to select, the double positive CD4+CD25+ T cells; finally to analyse intracellular FOXP3 staining on CD4+CD25+ T cells a histogram was created to analyse the mean fluorescence intensity (MFI) of FOXP3+ cells. Data are presented as dot-plots or histograms. Control stains were performed using isotype-matched mAb of unrelated specificity. Background staining was <1% and was subtracted from experimental values.

2.8. Determination of Soluble Cytokines. IL-1b, IL-6, IL-8, IL-10, IL-12p70, and TNF-α (Human Inflammation Cytokine Kit. BD Biosciences, Franklin Lakes, NJ, USA) were measured with cytometric bead arrays (CBA) in supernatants samples according to manufacturer's instructions (BD Biosciences) and analysed by flow cytometry with BD cytometric bead array software version 1.1.1 (Becton Dickson).

2.9. Statistical Analysis. Mann-Whitney U tests or Wilcoxon Rank Signed test was used to detect significant differences. The analysis was performed with Graphpad Prism software v.5.0. Differences were considered statistically significant when the test yielded P values less than 0.05.

3. Results

3.1. Frequency of CD25+ Cells and CD4+CD25+FOXP3+ Regulatory T Cells in Peripheral Blood Mononuclear Cells. We began by determining the percentage of CD25+ and CD4+CD25+ T cells in the peripheral blood of 14 patients with allergic conjunctivitis and 7 healthy controls. As expected, the percentage of CD4+ T cells were similar among patients with perennial allergic conjunctivitis (PAC) and healthy controls (HC) (MD 27%, IQR 24–30 versus MD 34%, IQR 28–35, resp.; $P = 0.07$); meanwhile, the frequency of CD25+ cells was significantly increased in patients with PAC compared to HC (MD 5%, IQR 2.9–8.2 versus MD 0.1%, IQR 0.07–1.5, resp.; $P = 0.001$). Likewise, the percentage of CD4+ T cells expressing CD25 were also significantly increased in patients with PAC in comparison to HC (MD 18.6%, IQR 7.2–22 versus MD 0.5%, IQR 0.4–1.7, resp.; $P = 0.0006$) (Figures 1(a) and 1(b)). When we analysed the frequency of FOXP3 on CD4+CD25+ gated cells, the majority of the CD25+ helper cells were FOXP3− in both PAC and HC groups (MD 3.4% IQR 1–7 versus MD 1.8% IQR 0.4–9, resp.; $P = 0.5$) (Figure 1(c)). Interestingly, MFI in FOXP3+ cells from patients with PAC was significantly decreased when it was compared with MFI in FOXP3+ cells from HC (MFI 17 ± 9 versus MFI 93 ± 13, resp.; $P < 0.0001$) (Figure 1(c)).

3.2. Frequency of Chemokine Receptors on Peripheral Blood Mononuclear Cells. To determine whether chemokine receptor expression was associated with a particular T helper cell traffic molecule in patients with PAC, CCR4, CCR7, and CCR9 was measured on PBMC. Results are summarized in Table 1. It was observed that CCR4+ cells were 1.9 times more frequent on PBMC from patients with PAC than in HC ($P = 0.004$). Most of the CCR4+ cells were CD4+ T cells, and CD4+CCR4+ cells were 1.8 times more frequent in patients with PAC than in HC ($P = 0.03$) (Figures 2(a) and 2(d)). We did not observe differences in frequency of CCR7+ or CCR7− cells neither on PBMC nor on CD4+ cells among groups (Figures 2(b) and 2(e)). The CCR9+ cells were 4.2 times more frequent on PBMC from patients with PAC than in HC ($P = 0.01$), and the CD4+ T cells expressing CCR9 were 2.5 times more frequent in patients with PAC than in HC ($P = 0.01$) (Figures 2(c) and 2(f)) (Table 1).

3.3. Frequency of CD4+CD25+FOXP3+ Regulatory T Cells and Cell-Migration Receptors after Dermatophagoides pteronyssinus (Der p)-Stimulation. To establish the potential involvement of the specific antigenic-stimulation in the expression of FOXP3 and in the upregulation of cell migration receptors in PBMC from patients with PAC, we assessed the percentage of CD4, CD25, FOXP3, CCR4, CCR7, CCR9, CD103, and CD108 after *Der p* stimulation in 7 patients with active perennial allergic conjunctivitis. The specific allergic condition to *Der p* was confirmed by a skin-prick test positive to *Der p* (wheal, >3 mm diameter) and determination of IgE specific to *Der p1* (49.8 ± 39.5 kU/L). After allergen-stimulation, we observed a significant increase in the percentage of CD25+ cells ($P = 0.0007$) and CD4+CD25+ cells ($P < 0.0001$) (Figures 3(a) and 3(b)); and although frequency of CD4+CD25+FOXP3+ regulatory T cells was increased 7-folds when compared with nonstimulated cells ($P < 0.0001$) (Figure 3(c)), we did not observe significant differences between the frequency of FOXP3+ versus that of FOXP3− subsets on gated CD4+CD25+ cells after specific stimuli (Figure 3(d)). Afterwards, we analysed the frequency of chemokine receptors positive cells, and 1.9 times more CCR9+ cells ($P = 0.04$) and 2.5 times more CD4+CCR4+CCR9+ cells were observed ($P = 0.01$) after *Der p*-stimulation (Table 2). No significant changes were observed in the percentage of the following cell subsets: CCR4+, CD4+CCR4+, CCR7+, CCR7-CD4+CCR7+, and CD4+CCR7− cells. Moreover, we observed 4.1-fold more CD4+CD103+ cells ($P = 0.007$) (Figure 4), 2.5-fold more CD108+ cells ($P = 0.01$), and 4.9-fold more CD4+CD108+ cells ($P = 0.01$) after allergen specific stimulation (Figure 5). Results are summarized in Table 2.

3.4. Cytokines after Der p-Stimulation. The levels of secreted cytokines IL-1b, IL-6, IL-8, IL-10, IL-12p70, and TNF-α were determined in culture supernatants after *Der p*-stimulation. IL-6 and IL-8 were significantly increased when compared with nonstimulated cells ($P = 0.01$ and $P = 0.04$, resp.). Results are depicted in Table 3. In order to know if

Table 1: Frequencies of chemokine receptors in patients with PAC and HC.

Chemokine receptor	Patients with PAC MD (IQR-Range)	Healthy controls MD (IQR-Range)	*P*
CCR4+	19% (15.8–40.6)	10% (9.6–14.2)	0.004
CCR7+	35% (17–47.7)	33% (29.2–37.4)	0.688
CCR7−	65% (52.3–83)	67% (62.6–70.8)	0.688
CCR9+	18% (8–29.8)	4% (3.9–6.5)	0.01
CD4+CCR4+	42% (31.1–53.8)	23% (20.8–43.8)	0.03
CD4+CCR7+	67% (45.6–77.6)	61% (24.8–76)	0.433
CD4+CCR7−	33% (22.4–54.4)	39% (24–75.2)	0.433
CD4+CCR9+	18% (8–29.8)	4% (3.8–6.5)	0.01

MD: Median, IQR: Interquartile range.

Table 2: Frequencies of cell-subsets and cell-migration receptors after *Der p*-stimulation.

Cell-subsets	RPMI MD (IQR-Range)	*Der p* MD (IQR-Range)	Con A mitogen MD (IQR-Range)	*P*	
				RPMI versus *Der p*	*Der p* versus Con A
CD25+	12.5% (10.9–15.7)	20.4% (16.0–26.4)	87.5% (78.2–93.4)	0.001	<0.0001
CD4+CD25+	16.2% (12.5–20.8)	28.2% (23–32.7)	99.4% (99–99.5)	0.0004	<0.0001
CD4+CD25+ F0XP3+	3.5% (1.8–14.9)	57% (50–63.5)	18% (12.8–28.4)	<0.0001	<0.0001
CCR4+	19% (13.2–28)	21% (16–27.6)	69% (56.6–80.2)	0.312	0.002
CCR7+	55% (41.5–63.5)	49% (36–56.9)	84% (72.1–88.2)	0.687	0.0006
CCR7−	45% (36.5–58.5)	51% (43.1–64)	16% (11.8–27.9)	0.687	0.0006
CCR9+	1% (0.5–2.2)	2% (1.9–9.9)	3.4% (2.9–7.6)	0.04	0.62
CD4+CCR4+	32% (21.6–50.7)	32% (24.8–45.3)	91% (84.7–97.5)	0.62	0.001
CD4+CCR7+	86% (79.7–89.1)	82% (74.2–86.9)	98% (95.0–98.5)	0.06	0.0006
CD4+CCR7−	14% (10.9–20.3)	18% (13.1–25.8)	2% (1.5–5.0)	0.06	0.0006
CD4+CCR9+	1% (0.6–3.4)	4% (2.5–19.5)	3.7% (2.9–6.6)	0.15	0.62
CD4+CCR4+ CCR9+	5.2% (2.5–5.5)	13% (10–42.7)	6% (5.4–9.8)	0.01	0.01
CD103+	4% (2.6–7.5)	6% (4.0–7.1)	33% (26.0–34.2)	0.295	0.001
CD108+	9% (30–14.4)	23% (15.6–35.5)	76% (63.8–76.7)	0.01	0.0003
CD4+CD103+	2% (0.9–2.8)	7% (3.3–11.0)	38% (13.8–57.2)	0.007	0.001
CD4+CD108+	6% (1.3–7.6)	27% (14.5–29.9)	76% (69.7–84.8)	0.01	0.002

MD: Median; IQR: Interquartile range.

Table 3: Cytokines concentration in supernatants of cell culture.

	RPMI MD (IQR-Range)	*Der p* MD (IQR-Range)	Con A MD (IQR-Range)	*P*	
				RPMI versus *Der p*	*Der p* versus Con A
IL-1β	55.6 (50.2 ± 63.6)	52.4 (44.3 ± 67.9)	115 (96.9 ± 205)	0.99	0.01
IL-6	118.9 (72.3–291.2)	480.6 (284 ± 515.3)	2554 (1724 ± 6239)	0.01	0.001
IL-8	13579 (11145 ± 14853)	14753 (14332 ± 18661)	12657 (11094 ± 14287)	0.04	0.01
IL-10	62.51 (55.9–104.9)	69.1 (60.8–175.4)	243 (173–324)	0.32	0.09
IL-12p70	51.6 (50.5–63.9)	48.1 (46.4–49.2)	4458 (2077–6951)	0.06	0.001
TNF-α	34.9 (33.0–61.8)	32.3 (31.23–34.7)	6942 (4070–19610)	0.07	0.0007

MD: Median; IQR: Interquartile range.
Results are in pg/mL; kit detection limits were as follows: IL-1β: 3.7 pg/mL; IL-6: 4.7 pg/mL; IL-8: 3.4 pg/mL; IL-10: 4.1; IL-12p70: 4.0; TNF-α: 3.9 pg/mL.

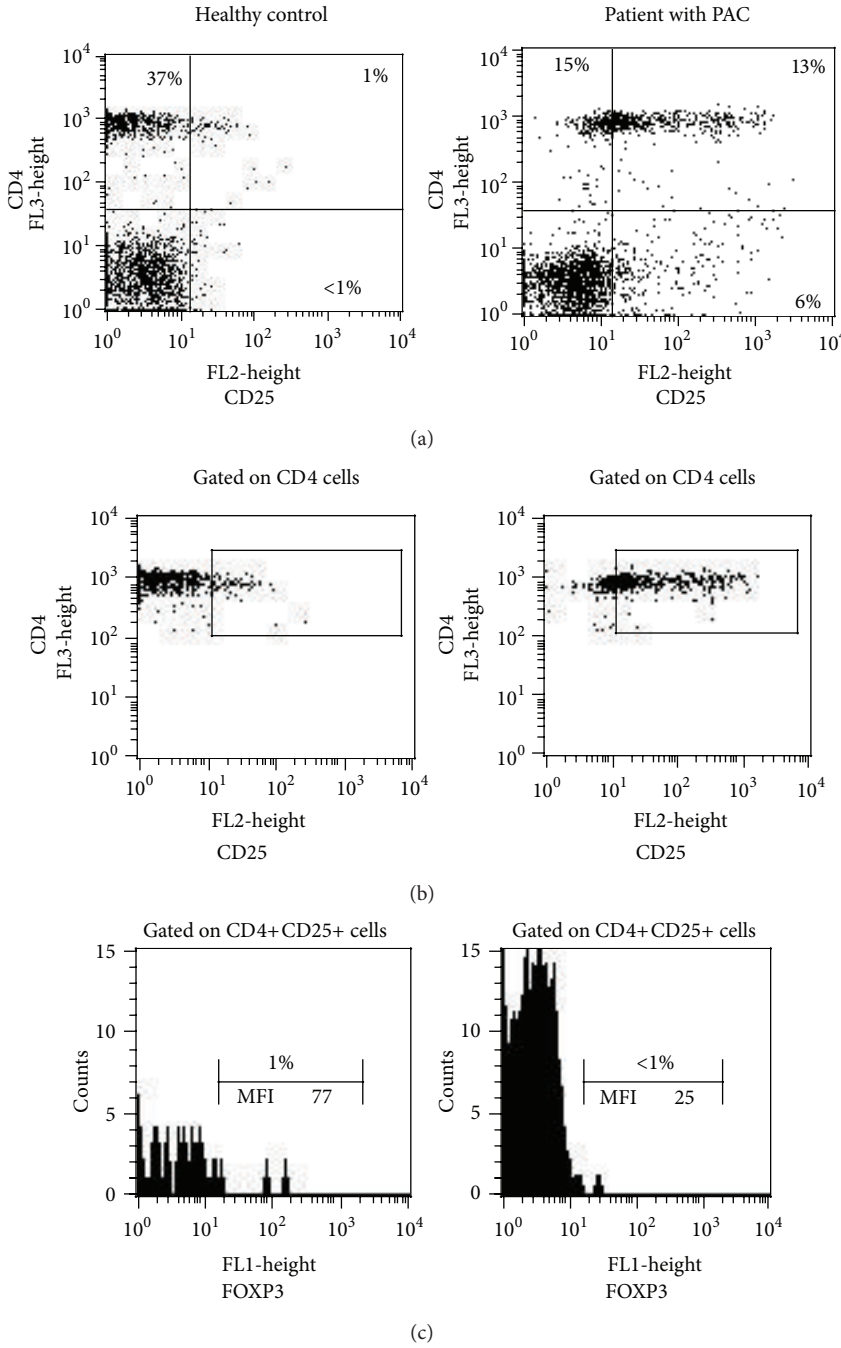

Figure 1: Frequency of CD25+ cells and CD4+CD25+FOXP3+ regulatory T cells in peripheral blood mononuclear cells. (a) Frequency of CD4+, CD25+, and double positive to CD4+CD25+ cells in PBMC from HC and PAC patient. (b) Same dot plot as (a); CD4+ cells gated as previously described in materials and methods, showing a gate performed to analyse CD4+CD25+ double positive cells. (c) Histogram from CD4+CD25+ gated cells (gated in (b)); the x-axis denotes FOXP3 (MFI = mean fluorescence intensity). These dot plots and histograms are representative of 7 HC and 14 PAC patients.

Der p-stimulation induced early secretion of IL-5 in CD4+ cells, we performed intracellular evaluation of IL-5 in CD4+ T cells from patients with PAC. We observed 10.3 times more frequency of CD4+IL-5+ cells after *Der-p* stimulation when compared with RPMI alone (Mean 15.5% SD 3.6 versus Mean 1.4% SD 3, resp.; $P = 0.02$) (Figure 6).

4. Discussion

Allergic conjunctivitis is an inflammation of the conjunctiva secondary to an immune response caused by contact with an allergen at the bulbar or tarsal conjunctiva in a previously sensitized individual [12]. Two types of AC have been described,

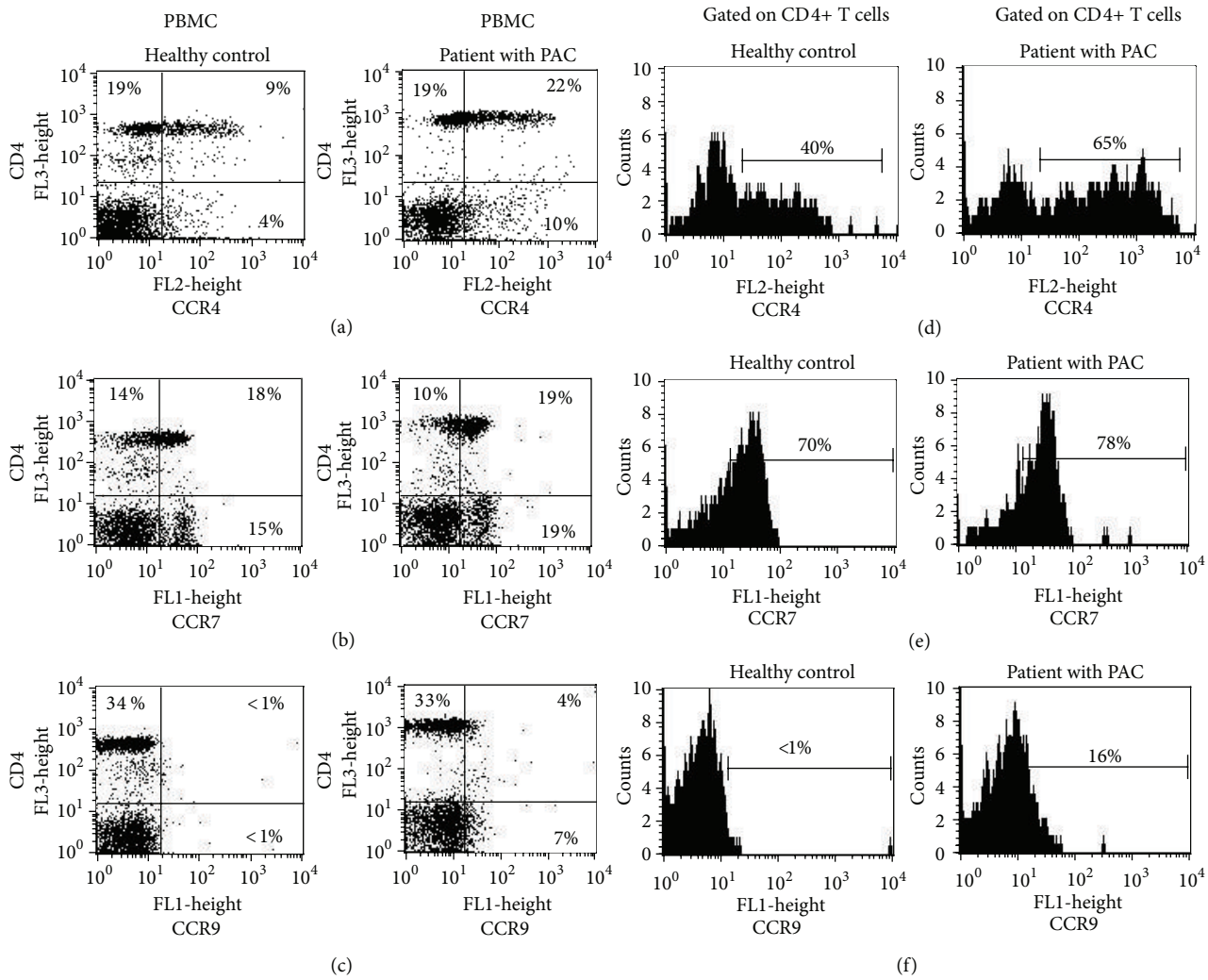

Figure 2: Frequency of chemokine receptors positive cells on circulating PBMC from healthy controls (HC) and from patients with perennial allergic conjunctivitis (PAC) PBMC were stained with fluorescence-conjugated antibodies to CD4 and (a) CCR4, (b) CCR7, and (c) CCR9, in a double-immunofluorescence assay, as described in materials and methods. Representative histograms of CD4+ gated cells are shown (d) CD4+CCR4+, (e) CD4+CCR7+, and (f) CD4+CCR9+ cells in both, HC and patients with PAC. Representative dot plots and histograms from 7 HC and 14 PAC.

the acute forms and the chronic forms; acute forms included SAC and PAC and are the most frequent types of AC and are clinically characterized by itching, redness, and tearing [2, 3, 12, 13]; VKC and AKC are the chronic forms and they could lead to permanent visual impairment due to persistent inflammation [13, 14]. In the chronic forms of AC, allergen-mediated inflammation is maintained by infiltrating CD4+ cells to conjunctiva [15]; migration of effector cells (T cells and non-T cells) is dependent of CCR3 and CXCR3 expression [10, 11]. In acute forms of AC, molecules involved in CD4 recruitment have not been enough studied. In mice models of AC, induction of CD4+CD25+FOXP3+ regulatory T cells suppresses effector-cell activation through synthesis of IL-10 and TGF-b [7]; nonetheless, the frequency of Tregs in patients with AC has not been described yet.

In this work we analysed the frequency of circulating Tregs and the frequency of cells expressing molecules involved in T-cell homing to mucosa inflammation, in patients with perennial allergic conjunctivitis. Our results are in accordance with other authors [16–19] that have reported changes in frequency of CD4+CD25+ T cells and CD4+CD25+FOXP3+ regulatory T cells in atopic diseases, such as asthma, rhinitis, and atopic dermatitis [16–19]. These authors suggest that changes in the frequency of Tregs or impairment of their regulatory capacity could be associated with the activation of allergic status. In this work, the majority of CD4+CD25+ cells were FOXP3− cells; and after allergen-stimulation, no differences were observed among the frequency of FOXP3+ cells and FOXP3− cells; this result is relevant since it is well known that IL-6 is able to suppress Treg differentiation [20]. It is possible that after encountering with the antigen, allergen-specific CD4+ T cells from PAC patients would be able to secrete IL-6, as it was observed after *in vitro* stimulation, interfering with CD4+CD25+FOXP3+

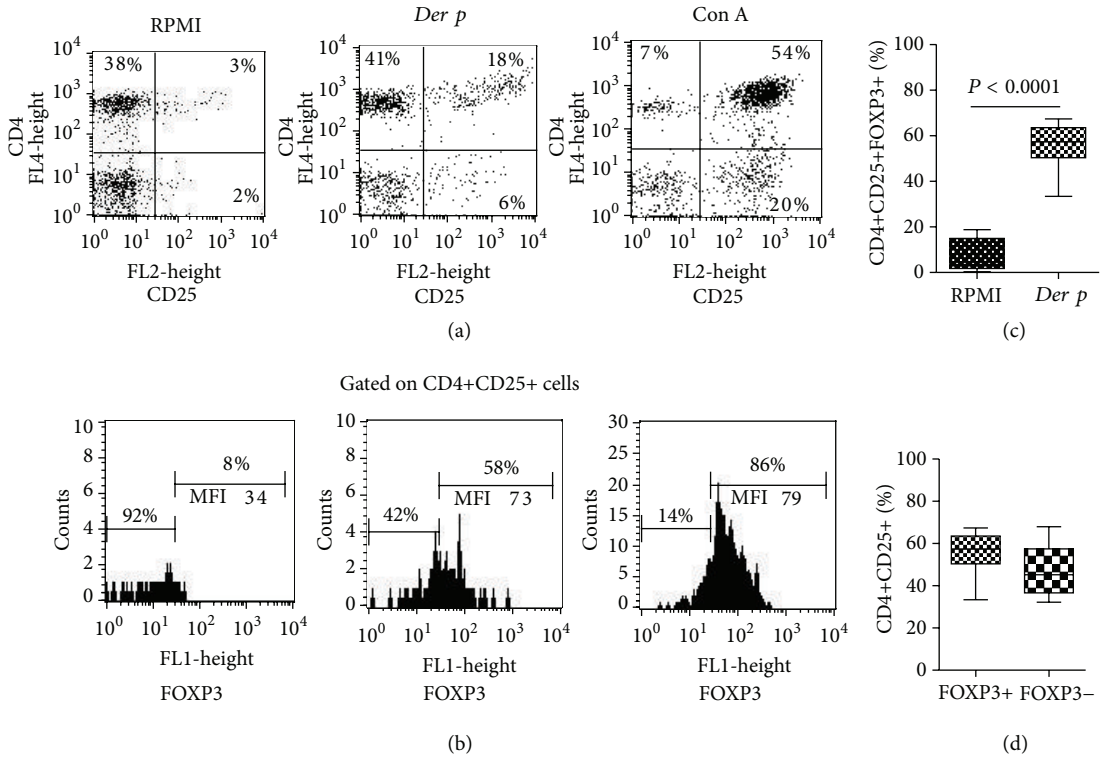

Figure 3: Frequency of CD25+ cells and CD4+CD25+FOXP3+ regulatory T cells after stimulation. (a) Representative dot plots of PBMC stimulated with *Der p* or Con A in patients with PAC. (b) Comparative histograms of FOXP3+ frequency on CD4+CD25+ gated cells. The *x*-axis denotes the mean fluorescence intensity (MFI) of FOXP3. (c) Significant differences were observed in the frequency of Tregs upon *Der p*-stimulation. (d) Distribution of FOXP3+ and FOXP3− cell subsets on CD4+CD25+ gated cells after allergen-stimulation.

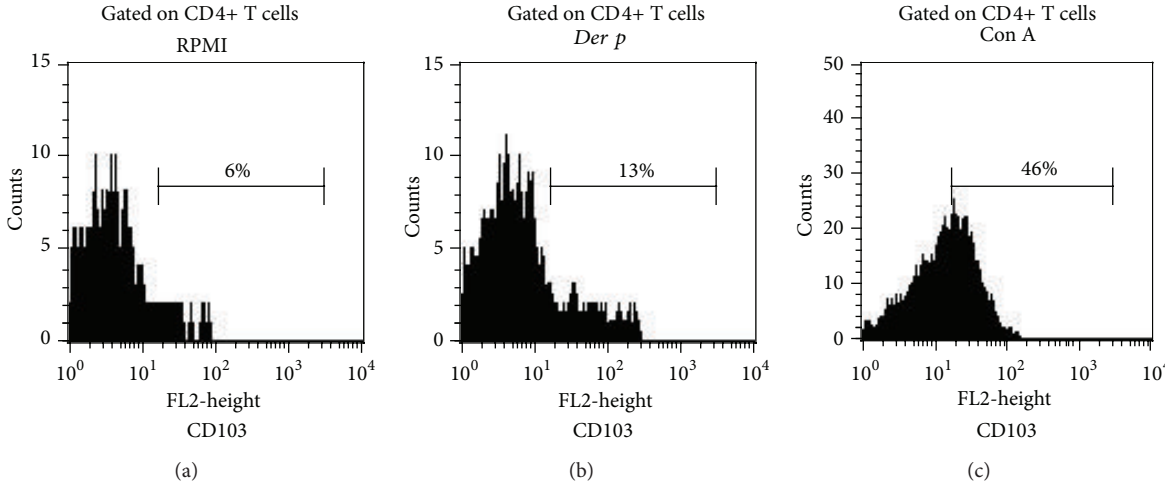

Figure 4: Increased frequency of CD4+CD103+ T cells after allergen-stimulation. (a) RPMI (nonstimulated cells, culture medium). (b) *Der p*-stimuli. (c) Con A mitogen (Positive stimulation control); *x*-axis denotes frequency of CD103+ cells on CD4+ gated cells from patients with PAC. Representative histograms of CD4+CD103+ T cells are shown.

differentiation, since it has been described that Treg cell differentiation requires antigen stimulation by engagement T cell receptor to induce FOXP3 expression [21]. Supporting this idea, the induction of FOXP3 by Con A-stimulation could be explained because of the polyclonal activation through mannose ligands on PBMC by Con A [22, 23]; nevertheless, FOXP3+ cells induced by Con A are mainly NnTregs, a different T cell subset of Tregs [24, 25].

On the other hand, it has been described that CD25 is the alpha subunit of IL2R and its expression has been related to

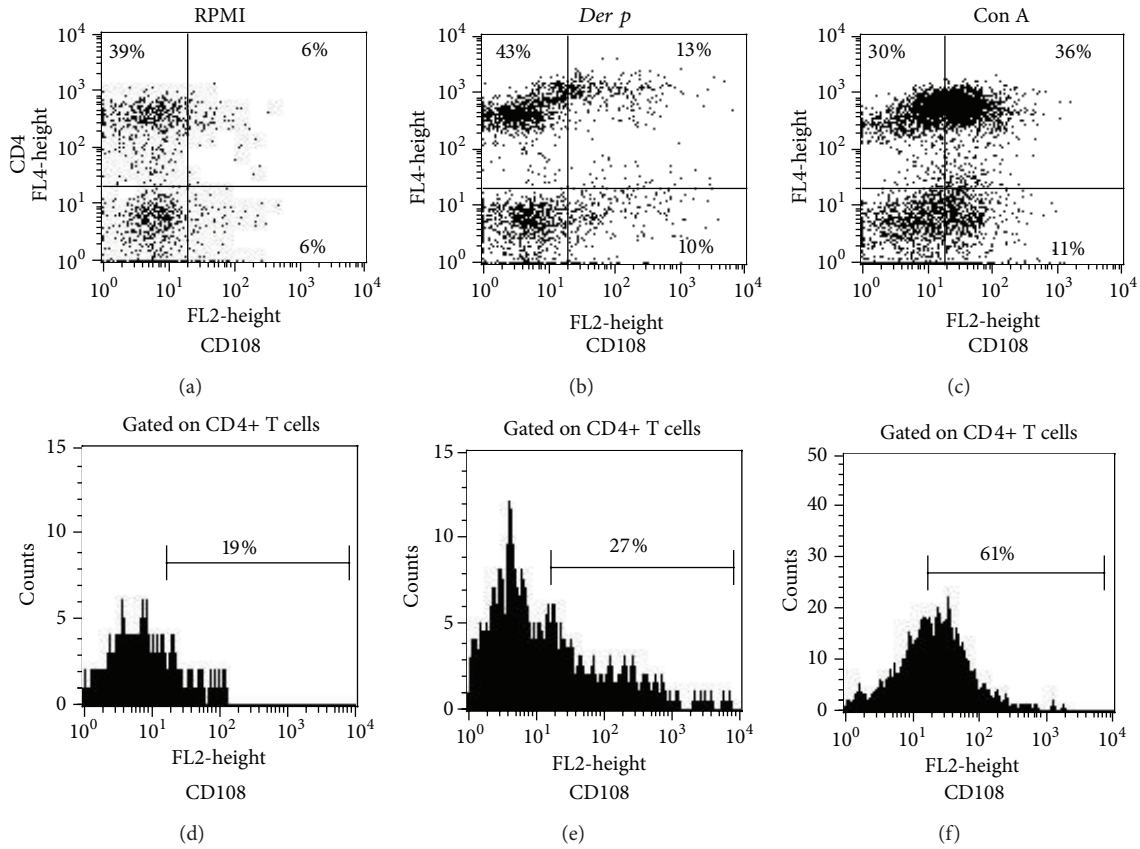

Figure 5: Increased frequency of CD108+ cells after allergen-stimulation. Representative dot plots of (a) nonstimulated cells, (b) *Der p*-stimulated cells, and (c) Con A-stimulated cells. Representative histograms of CD4+ gated cells, (d) nonstimulated cells, (e) *Der p*-stimulated cells, and (f) Con A-stimulated cells; *x*-axis denotes fluorescence to CD108 on CD4+ gated cells.

activation status of T cells [26]; interestingly, in this study, the frequency of PBMC expressing this cell surface marker was significantly elevated in PAC patients group in contrast to healthy individuals, suggesting that PBMC and circulating CD4+ T cells are in an activation status, as similarly reported in other allergies like asthma [27, 28] rhinitis [28], and dermatitis [29].

A differential expression of chemokine receptors was observed in patients with PAC; most CD4+ T cells were CCR4+ cells, and CCR9+ cells. These findings are remarkable, as CCR4 is known to modulate T-cell migration to sites of allergic-mediated inflammation in asthma and rhinitis [30, 31]; CCR4+ cells are also an important source of IL-4 and other Th2 cytokines [30, 31]. CCR9 is a molecule expressed on antigen-experienced memory T cells and it was described as a chemokine marker related to mucosal-homing [32]. It is possible that circulating CD4+CCR4 and/or CD4+CCR9+ cells observed in PBMC from our patients are cell-subsets in transit to conjunctiva, since after allergen-stimulation increased significantly the percentage of CD4+CCR4+CCR9+ cells. Remarkably, IL-4 is required for CCR9 imprinting on CD4+ T cells [33], and it is recognized that IL-4 and IL-5 are induced after *Der p* stimulation in allergic patients and promote allergic status [5].

HML-1 or CD103 ($\alpha_E\beta_7$ integrin) was first described as a molecule related to mucosal migration [34], and the vast majority of intraepithelial lymphocytes are CD103+ cells [9]. HML-1 could be induced by epithelial cells on activated lymphocytes [35] and has been implicated in epithelial T-cell retention through binding to E-cadherin [36]. In the present study, upon allergen specific-stimulation it was observed an increased percentage of CD4+CD103+ T cells. The CD4+CD103+ cells have been proposed as regulatory T cell subset [37]. Likewise, after *Der p* stimulation, the percentage of CD108+ cells were significantly increased. The CD108 (Sema7a) is a glycosylphosphatidylinositol-anchored semaphorin and has been described as a molecule that initiates T-cell-mediated inflammatory response through interaction with alpha1beta1 integrin [38]. On the other hand, it has been proposed that CD108 exists as a complex with TCR and/or CD3 on T cell surface and after activation inhibits T cell signalling and decreases proliferation [39]. In this context, whether the expansion of these two subsets, CD4+CD103+ cells or CD4+CD108+ cells, is related to

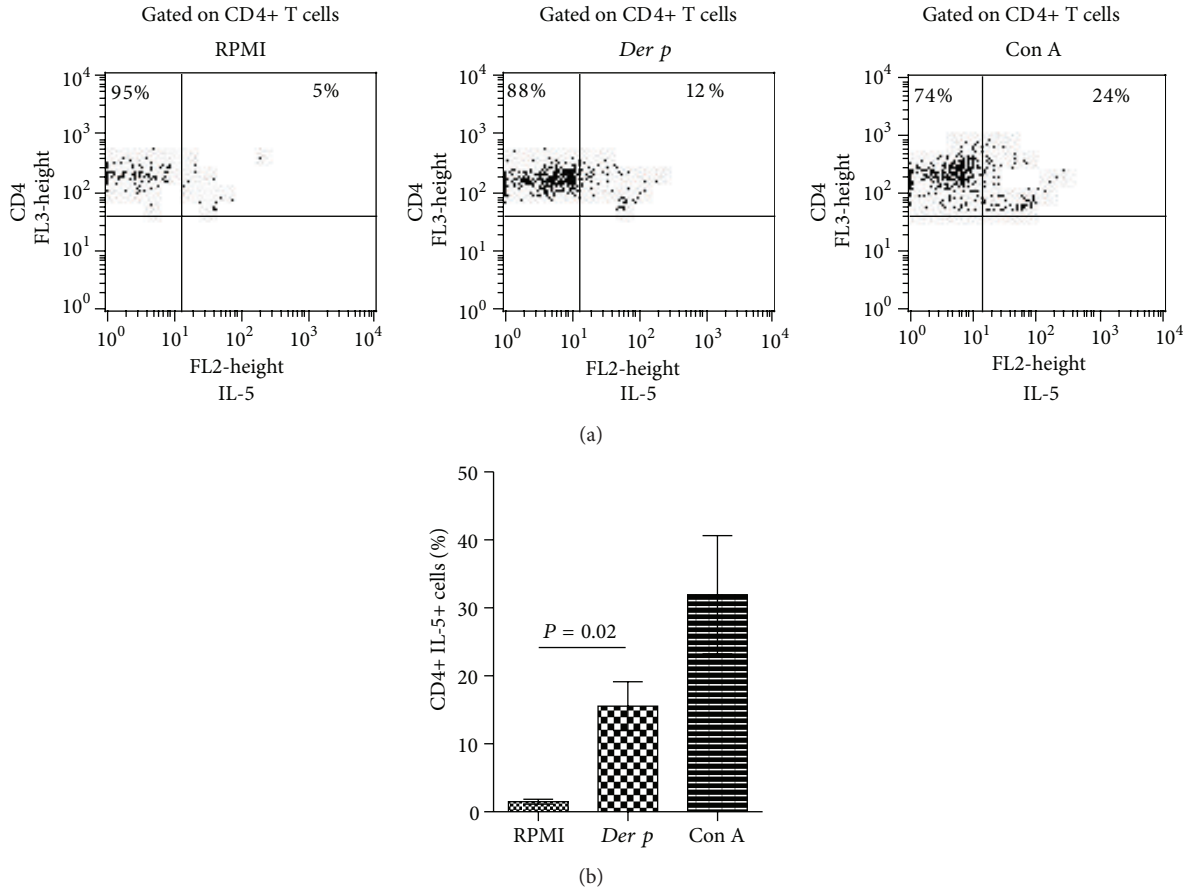

Figure 6: Frequency of CD4+ IL-5+ cells after *Der p*-stimulation. PBMC were *Der p*-stimulated for 72 h and stained for CD4 and intracellular IL-5 as described in materials and methods. (a) Left panel, nonstimulated cells, central panel, *Der p*-stimulated cells and right panel, and Con A-stimulated cells; representative dot plots from three PAC patients. (b) Comparison of the frequency of CD4+IL-5+ cells among different stimuli. Data are expressed as mean ± SD.

mucosa-homing or corresponds to regulatory T cell subsets trying to counterbalance inflammatory CCR4+CCR9+ cell subsets and IL-6/IL-8 secretion is not known and needs further investigation.

The data shown here could lead to new perspectives in the treatment of the most frequent ocular condition seen by ophthalmologists and allergo/immunologists; CCR4 and CCR9 molecules could be used as potential targets for biological therapy in patients with PAC, as it has been proposed for asthma and the monoclonal anti-CCR4 antibody [40].

Taken together, it is possible that the interaction of CCR4, CCR9, and possibly CD103 and CD108 with their ligands secreted or expressed on activated endothelial cells and conjunctiva favours the selective adhesion of a circulating CD4+ T cell subset with an activated phenotype and the ability to respond to antigens, driving the immune-response to the ocular mucosa and inducing a proinflammatory microenvironment related to Th2 perennial allergic conjunctivitis.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Sleep Loss as a Factor to Induce Cellular and Molecular Inflammatory Variations

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A reduction in the amount of time spent sleeping occurs chronically in modern society. Clinical and experimental studies in humans and animal models have shown that immune function is impaired when sleep loss is experienced. Sleep loss exerts a strong regulatory influence on peripheral levels of inflammatory mediators of the immune response. An increasing number of research projects support the existence of reciprocal regulation between sleep and low-intensity inflammatory response. Recent studies show that sleep deficient humans and rodents exhibit a proinflammatory component; therefore, sleep loss is considered as a risk factor for developing cardiovascular, metabolic, and neurodegenerative diseases (e.g., diabetes, Alzheimer's disease, and multiple sclerosis). Circulating levels of proinflammatory mediators depend on the intensity and duration of the method employed to induce sleep loss. Recognizing the fact that the concentration of proinflammatory mediators is different between acute and chronic sleep-loss may expand the understanding of the relationship between sleep and the immune response. The aim of this review is to integrate data from recent published reports (2002–2013) on the effects of sleep loss on the immune response. This review may allow readers to have an integrated view of the mechanisms involved in central and peripheral deficits induced by sleep loss.

1. Introduction

Sleep is a vital phenomenon, classically divided into two distinct phases: sleep with rapid eye movements (REM) and sleep without rapid eye movements (non-REM) [1]. In humans, three stages of non-REM sleep have been characterized by electroencephalography (EEG); these include low-frequency slow wave sleep (SWS) with EEG synchronization, light sleep, and an intermediate sleep stage 2. In contrast, REM sleep is characterized by EEG activity similar to that of waking and by the loss of muscle tone [2, 3]. Both phases, REM sleep and non-REM sleep, alternate throughout total sleep time [2, 3]. REM sleep is amply studied because it is considered important for learning, memory consolidation, neurogenesis, and regulation of the blood-brain barrier function [4–8], while non-REM sleep is related to hormonal release (e.g., growth hormone secretion), the decline in the thermal set point, and is characterized by a reduction of cardiovascular parameters (e.g., lowering of blood pressure) [9, 10]. Although sleep constitutes a considerable portion of the mammalian lifetime [2], specific sleep function still remains controversial. Many hypotheses have been proposed, including tissue repair, thermoregulation, homeostatic restoration, memory consolidation processes, and preservation of neuro-immune-endocrine integrity [10, 11].

The paramount role of sleep in the physiology of animal models and humans is evident by the effects of sleep loss. Serious physiological consequences of sleep loss include

emotional reactivity, cognitive dysfunction (deficits in learning, memory, and decision making), decreased neurogenesis, and metabolic disturbances that may result in the death of experimental animals [1, 7, 12–14]. Sleep loss effects can be evaluated by several methodologies, including acute total or selective sleep deprivation and sleep restriction (also called partial sleep deprivation) or sleep fragmentation. In some cases, deprivation devices connected to the electroencephalograph have been used to selectively deprive a specific sleep phase. In humans, total sleep deprivation is common in individuals working more than 24 hours continuously, while sleep restriction is defined as diminution of time spent asleep. Sleep restriction is linked to lifestyle including longer work hours and shift-work and increased accessibility to media of all sorts, or medical conditions such as insomnia [15, 16]. Pathological conditions (e.g., obstructive sleep apnea (OSA), drug addiction) and aging have a common pattern of sleep fragmentation (also called sleep disruption) characterized by numerous awakenings despite normal time spent asleep [16]. Most of the current knowledge on the effects of sleep loss in humans comes from studies of total sleep deprivation applied for brief time periods or partial sleep deprivation (2-3 hours less than normal sleep time) for one night or even for chronic periods [15, 16]. The majority of animal models used to study the physiological effects of sleep loss are based primarily on total sleep deprivation [16]. Although this method does not resemble human conditions, it still provides valuable information on sleep loss effects.

To study the relationship between sleep and the immune system, researchers have relied on two basic approaches; in the first approach, human volunteers or animals (mainly rodents) are subjected to the administration of immune-stimulating substances (or pathogen administration in animals), and the effects of these manipulations on sleep are evaluated. In the second approach, human volunteers or animals are subjected to sleep loss protocols (sleep deprivation, sleep restriction, or sleep fragmentation) and immunological products such as cells and/or soluble mediators are measured. Here, we present a compilation of recent evidence about the effects of sleep loss on the immune system in both humans and rodents, under acute and chronic sleep loss. Additionally, we propose how sleep recovery might restore the normal balance between proinflammatory and anti-inflammatory molecules at the systemic level and how immune mediators might be in direct contact with the central nervous system via blood-brain barrier disruption, modifying neural activity and the possible pathway for neurological impairments.

2. Sleep Loss as a Stressful Factor

Sleep loss has been deemed a stressor [17, 18]; however, sleep and stress differ in the profile of circulating molecules and in their effects on the immune system. Stress is the response of the organism to any stimulus that alters the homeostasis [19]. The adverse stimuli generating stress, either physical or psychological, also vary in their temporal dimension. Acute stress occurs when stressors appear once and remain for a short period of time (some minutes or hours); while, chronic stress occurs when stressors are repetitive and long lasting (appearing for weeks or months) [19]. Since the initial description of the phenomenon [20], it has been shown that stressors induce activation of the hypothalamus-pituitary-adrenal (HPA) axis and of the sympathetic nervous system [19, 21]. At the beginning of the stress response, there is a large sympathetic activation, followed by glucocorticoid release from the adrenal cortex. Over a prolonged stress period, the adrenaline response is rapidly habituated; however, glucocorticoids remain elevated only when stressors are unpredictable and uncontrollable. If the subject is capable of predicting the appearance of chronic stressors and has control over them, the glucocorticoid response also disappears [21].

Regarding the effect of stress on the immune system, it has been shown that acute stress has an immunostimulatory effect; adrenaline increases the circulating numbers of neutrophils, macrophages, natural killers, and lymphocytes, while glucocorticoids promote traffic of leukocytes to the skin, mucosal lining of the gastrointestinal and urinary-genital tracts, the lung, and liver, both in humans and in experimental animal models [22–27]. Therefore, acute stress seems to prepare the immune system to cope with the damage induced by the noxious agent. On the contrary, chronic stress suppresses the immune function by modifying the levels of both proinflammatory (e.g., interleukin (IL)-6 and tumor necrosis factor (TNF)-α) and anti-inflammatory cytokines (e.g., IL-10, IL-4) [28], by reducing the numbers and traffic of leukocytes [27], and by up-/downregulating T cell number and function [29]. Specifically, glucocorticoids act on antigen-presenting cells (APCs) and T helper 1 (T_h1) cells, inhibiting their production of IL-12, interferon (IFN)-α, IFN-β, and TNF-α, but upregulating the production of anti-inflammatory cytokines (IL-4, IL-10, and IL-13) by T_h2 cells [30].

Since the pioneer studies, sleep loss has been tightly linked to stress; in the first studies it was shown that sleep deprived animals had larger adrenals than their counterpart controls [1, 31]. In animal models, the classical methods for sleep deprivation consist of highly aversive environments (e.g., water surrounding small platforms); therefore, additional animals subjected to the aversive environment but without any sleep loss are constantly included as controls for the procedure. The measurement of circulating levels of glucocorticoids is the main stress index; nevertheless, depending on the intensity and duration of sleep loss, cortisol/corticosterone levels may increase [32–35], not change [33, 36], or even decrease [37] (see Table 1). It is known that the chronic increase in cortisol/corticosterone levels desensitize glucocorticoid receptors, promoting an altered control of the HPA axis [38]; this may explain the maintenance or even the decrease in glucocorticoid levels after sleep deprivation (e.g., >40 h in humans) [39] or chronic sleep restriction (e.g., 21 days in rats) [33].

The role of glucocorticoids in sleep homeostasis has been carefully studied; glucocorticoid administration in both humans and animal models induces waking EEG activity (e.g., [42, 43]); in addition, glucocorticoid administration decreases REM sleep and promotes SWS in humans [42] and decreases SWS and increases sleep latency in animal

Table 1: Differential effect of sleep loss time upon glucocorticoid levels.

Human	Cortisol	Reference
TSD 1 night	↑	[34]
TSD 40 hours	= or ↓	[40]
TSD 40 hours	=	[39]
SR 2 hours TIB/1 night	=	[36]
SR 3 hours TIB/4 days	↓	[37]
SR 6 hours TIB/6 days	=	[41]
Rodents	Costicosterone	Reference
RSD 72 hours	↑	[32]
RSD 96 hours	↑	[33]
RSR with 6 hours of SO/21 days	=	[33]

The table illustrates the differential effect of acute sleep deprivation and sleep restriction upon glucocorticoid levels. Representative samples present in this table were measured within the first four hours after wakefulness in humans or at the beginning of the light phase in rodents.
Abbreviations: TSD: total sleep deprivation; SR: sleep restriction; TIB: time in bed; RSD: REM sleep deprivation; SO: sleep opportunity; ↑: increase; =: not change; ↓: decrease.

models [42]. When they occur, increased corticosterone levels secondary to sleep deprivation are unnecessary for sleep recovery; in animal models, a large sleep rebound was observed after acute sleep deprivation, despite adrenalectomy [44]. Moreover, under chronic REM sleep deprivation in rats, where corticosterone levels are similar to basal levels [33], a tendency to REM sleep rebound is also observed [45].

In the vast majority of studied phenomena (e.g., studies of sleep loss effects on hippocampal neurogenesis), it has also been found that sleep loss effects are maintained even in animals subjected to adrenalectomy [46]. Additionally, chronic administration of an inhibitor of corticosterone secretion (metyrapone) in REM sleep deprived animals did not revert memory deficits; hence glucocorticoids are not responsible for the memory impairments associated to REM sleep loss [47]. These data show that sleep loss may cause more functional deficits than those caused by stress only. It is very likely that the effects of REM sleep deprivation on neural, endocrine, and immune systems accumulate throughout the experimental procedure without any opportunity to restore homeostasis by adequate sleep recovery. Notwithstanding, some authors still consider that sleep loss is a stressful event [18], while the vast majority of sleep researchers deem sleep deprivation and stress as independent events [42–44, 46–48].

3. Sleep and the Immune Response

It is well known that sleep loss makes an individual more susceptible to disease and, conversely, that sleep is important for recovery from illness. Specific immunological active peptides or neuroendocrine hormones influence the sleeping-waking brain, and sleep disturbances may affect inflammatory components. Cellular (macrophages, neutrophils, eosinophils, basophils, natural killer, and T and B lymphocytes) and molecular (proinflammatory cytokines and acute phase proteins) inflammatory components that act as mediators of the acute phase response in inflammatory diseases, additionally, play a role as modulators of metabolic functions that involve the central nervous system, including sleep.

3.1. Effects of Inflammatory Components on Sleep. Cytokines that affect sleep in both humans and laboratory animals include IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-15, IL-18, TNF-α, TNF-β, IFN-α, IFN-β, IFN-γ, and macrophage inhibitory protein (MIP)-1β (MIP-1β) [49]. Immune signaling molecules such as cytokines are present in the healthy brain, where they interact with neurochemical systems (e.g., serotoninergic, cholinergic, and glutamatergic systems) [49, 50] to regulate normal sleep. Particularly, IL-1α, IL-1β, and TNF-α have been widely investigated to state that they are involved in the regulation of physiological sleep. Signaling receptors for both IL-1 (α and β) and TNF-α are present in brain areas involved in sleep physiology including the hypothalamus, brainstem, hippocampus, and cerebral cortex [49]. The brain interacts with peripheral inflammatory mediators through the innervation of lymphoid tissues or the transport or action of these molecules on the blood-brain barrier [51]. In addition, glial cells such as microglia and astroglia, as well as pericytes are capable of releasing proinflammatory mediators in response to peripheral signals (chemokines, acute phase proteins, nitric oxide, and adenosine) contributing to the action of inflammatory mediators upon neuronal function [52, 53]. Because IL-1α, IL-1β, and TNF-α are the most studied cytokines involved in sleep regulation, we focus mainly on these three cytokines; however, the role of IL-6 will also be reviewed because this proinflammatory cytokine is highly related to the interaction between sleep loss and the immune response.

3.1.1. Effect of Proinflammatory Cytokine on Sleep in Humans. Interleukin-1 is a key mediator of the acute phase response in an infected host [54]. IL-1α and IL-1β together with TNF-α have many physiological roles, such as in cognition, synaptic plasticity, and immune function. Both IL-1β and TNF-α are also well-characterized as to their actions on sleep regulation [55]. For instance, IL-1β is a potent enhancer of non-REM

sleep that induces symptoms associated with sleep loss such as sleepiness, fatigue, and poor cognition [56].

Under pathological conditions (e.g., cancer, multiple sclerosis) cytokine administration is used as a treatment [57, 58] and sleep patterns are altered [59, 60]; in patients with multiple sclerosis numerous sleep pathologies (e.g., insomnia, hypersomnia, circadian rhythm sleep disorders, and movement- and breathing-related sleep disorders) have been described [59], while in cancer patients complaints about sleep fragmentation and insomnia are frequent [60]. Although sleep disturbances are frequently reported in autoimmune pathologies and mood disorders with an inflammatory component [59–61], the aetiology of sleep alterations remains unclear. To cite a few instances, it has been reported that autoimmune diseases that exhibit autoantibodies against neuronal voltage-gated potassium channel (VGKC) complexes such as limbic encephalitis or Morvan syndrome present sleep disturbances like insomnia, REM sleep behavior disorder, hypersomnia, and somniloquy [62, 63]. Interestingly, immunotherapy in patients with autoimmune diseases promotes significant sleep improvement in 80% of patients [62]. Also, infections with a proinflammatory component induce sleep disorders, up to 70% of persons living with human immunodeficiency virus (HIV) experience sleep disturbances including insomnia and obstructive sleep apnoea (OSA) syndrome [64], and in people affected by leprosy the prevalence of restless leg syndrome is higher than the general population [65]. In the same way, inhibition of proinflammatory cytokine signalling has been proposed as a viable strategy for targeting sleep disturbances in patients with evidence of proinflammatory activity [66]. For instance, in alcohol-dependent males, inflammatory markers correlated with REM sleep increase [66], but the pharmacological neutralization of TNF-α by etanercept (a decoy receptor that binds to TNF-α) reduced REM sleep until normal values [67]. In addition, both IL-1 (α and β) and TNF-α are present in a variety of clinical conditions involving sleep disorders, such as chronic insomnia and OSA (reviewed in [68]).

3.1.2. Effect of Proinflammatory Cytokines on Sleep in Animal Models. It has been known for over 50 years that mammalian cerebrospinal fluid contains sleep-promoting substances that accumulate during wakefulness [10]. The common criteria to consider any substance a somnogenic molecule include (1) whether the substance injected enhances sleep, (2) whether sleep is reduced if the substance is inhibited, and (3) whether the substance is altered in pathological states associated with sleep disorders. All of these criteria have been met by IL-1α, IL-1β, IL-6, and TNF-α [68].

The effects of IL-1α, IL-1β, and TNF-α on sleep was reported in several animal species including rodents, monkeys, cats, and sheep. Induction of non-REM sleep by IL-1α, IL-1β, and TNF-α is independent of the route of administration (e.g., intracerebroventricular (ICV), intraperitoneal, subcutaneous) and its effect is dose-dependent [68, 69]. In rodents, classical studies show that low doses of IL-1β, through ICV administration, increase non-REM sleep when it is administered during the light phase [69]. However, IL-1α or IL-1β also induce non-REM sleep fragmentation [49], and high doses of IL-1β, administered during the dark phase, suppress non-REM sleep [68].

In addition to the pioneer studies on sleep regulation by cytokines, recent studies focus on the molecular pathways involved in physiological sleep regulation. Recently, mice lacking the TNF 55kDa receptor (TNFR-KO) present a decrease in the amount of non-REM and REM sleep [70]. Furthermore, experimental studies in rodents show that proinflammatory cytokine-induced sleep disturbances can be reversed by administration of anti-inflammatory cytokines or specific cytokine antagonists (e.g., IL-1 receptor antagonist, IL-1ra) [68]. The strong relationship between sleep and its modulation by proinflammatory cytokines provides a key to understand how sleep loss is capable of altering the immune system and subsequently promotes metabolic, cardiovascular, and neurodegenerative impairments [15].

3.2. Effect of Sleep Loss on Immunological Response in Humans

3.2.1. Effects of Sleep Loss on Cellular Immune Components. Circadian rhythms have been described for white blood cells (WBC) in humans; numbers of circulating natural killers (NK) and neutrophils peak at midday and show a nadir during the night; while, monocytes, T and B lymphocytes peak during the first half of the night and present the lowest values during the day hours [71]. Sleep loss shifts the normal circadian rhythm of WBC. In 24-hour total sleep deprived humans, monocytes, T and B lymphocytes presented a delay in the zenith of the rhythm with attainment of peak values between 3 and 6 hours later than in normal sleep conditions [71]; while the rhythm of NK flattened with a net increase in the NK number during the sleep deprived night as compared to normal sleep conditions [71, 72]. However, only few human studies have repeatedly drawn blood samples from sleep deprived subjects to measure circadian effects of sleep deprivation on WBC counts; the majority of reported studies quantify circulating WBC only once, on the morning after sleep deprivation and compare those values with normally sleeping subjects. Generally, in those studies leukocyte population increases after acute sleep deprivation, mainly by rises in circulating numbers of monocytes and neutrophils; in contrast, circulating numbers of B and T lymphocytes remain stable immediately after sleep loss, but exhibit changes after sleep recovery (see Table 2) [73, 74]. Sleep restriction to 4 hours in bed during 5 consecutive nights decreased the number of circulating NK and increased the number of B lymphocytes, maintaining stable the numbers of other WBC [75]. Differences among these studies may be explained by the different techniques to draw blood samples, such as sex, race, or age of the participants.

3.2.2. Effects of Sleep Loss on Molecular Inflammatory Component

Effect of Sleep Loss on Antibodies. Few studies have examined the consequences of sleep loss on the immune response to

Table 2: Sleep loss effects on immune cellular components in humans.

Sleep loss condition	Subject's characteristics	Cells		Reference country
Sleep deprivation	11 males	Leukocyte ↑*	B lymphocytes =	[73]
2 nights	19–29 years	Neutrophil ↑**	T lymphocytes =	Brazil
Sleep restriction	10 females	WBC ↑**	B lymphocytes =	[74]
4 hours time in bed	PM-RT	Monocytes ↑*	T lymphocytes =	Belgium
3 nights	55–65 years	Neutrophils ↑*		
Sleep restriction	7 females, 7 males	NK =	B lymphocytes =	[76]
4.5 hours time in bed	39–61 years	Monocytes =	T lymphocytes =	USA
1 night				
Sleep restriction	8 males	Neutrophils =	Lymphocytes =	[77]
4 hours time in bed	22–29 years			Belgium
3 nights				
Sleep restriction	13 males	Monocytes =	B lymphocytes ↑**	[75]
4 hours time in bed	19–29 years	NK-cells↓**	T lymphocytes =	Finland
5 nights				

The table illustrates the differences between sleep deprivation and sleep restriction upon cellular components of the immune system in humans.
Abbreviations: NK: natural killers; PM-RT: postmenopausal with replacement therapy; ↑: increase; =: not change; ↓: decrease; *significant differences with $P < 0.05$; **significant differences with $P < 0.01$.

vaccination in healthy individuals; highly variable findings have been reported [78–80]. Total sleep deprivation during one night prior to hepatitis A vaccination reduced specific antibody titters in the long-term (28 days postvaccination) in both males and females [78]. However, the same 24 hours of total sleep deprivation reduced specific antibody titters to influenza AH1N1 virus vaccine only in males in the short term (5 days post-vaccination), while sleep deprived females did not have a significant difference as compared to normal sleeping subjects [79]. In another study, short sleep durations during the week of hepatitis B vaccination decreased viral specific antibody titters in both male and female volunteers; while the contrary was true, higher levels of antibody titters were observed in participants with long sleep durations during the week of vaccination [80]. Although few, those studies suggest that sleep plays an important role in humoral immunity, especially in antibody production; however, more studies are necessary to elucidate how sleep loss may induce changes in cellular immune components and subsequently induce antigen-specific immune impairments, such as insufficient antibody production.

IL-1α, IL-1β, IL-6, and TNF-α: The Most Studied Cytokines under Sleep Loss Conditions. Human studies that evaluate sleep loss effects have focused on the correlation among inflammatory markers and metabolic and cardiovascular diseases. For instance, in a study with 124 healthy volunteers, inflammatory markers, such as endothelin-1 (ET-1) and IL-6, were associated with an increase in total sleep time and REM sleep latency [81]. These results show that poor sleep is directly associated with inflammatory status. In the same way, shorter sleep duration is also related to obesity and cardiovascular diseases [82]. It is known that obesity, diabetes, and cardiovascular diseases share a common mechanism characterized by the inflammatory process. If sleep loss induces low-intensity inflammation, we may consider that sleep loss is associated with metabolic and cardiovascular disease generation through immunological deregulation [15].

Similar to immune cells, cytokine production presents circadian rhythms; proinflammatory cytokines present a peak in early nocturnal sleep in correlation with the accumulation of molecules such as adenosine or reactive oxygen species that promote proinflammatory cytokine release; however the dominance of the proinflammatory response shifts during late sleep, when REM sleep is present, promoting the production of anti-inflammatory cytokines [51, 83]. The different periods of exposure to proinflammatory mediators might explain the reported differences between cytokine plasma levels in sleep loss protocols.

Sleep deprivation protocols, lasting 40–88 hours in humans, induce controversial changes in plasma levels of IL-1α, IL-1β, IL-6, and TNF-α, with reported findings of increases, decreases, or absence of measurable changes in cytokine levels [34, 39–41, 83, 84] (see Table 3). For example, IL-6 plasma levels increased after one week of sleep restriction in healthy males [41]. In contrast, a study with 40 hours of continuous total sleep deprivation found decreased IL-6 levels in healthy men [40]. These discrepancies may be attributed to the method employed to obtain blood samples; intravenous catheters used for repetitive blood sampling increase local IL-6 production, which might confound the sleep-dependent changes in plasma concentrations of this cytokine [85]. In addition, all the cellular sources of proinflammatory cytokines are not known, although monocytes, which make up about 5% of circulating leukocytes, are major contributors

Table 3: Sleep loss effects on immune molecular inflammatory mediators.

Sleep loss condition	Subject's characteristics	Cytokines (pg/mL)	C-reactive protein	Reference
Total sleep deprivation 1 night	16 controls 11 females, 5 males BMI 20.7–24.1 kg/m^2 15 unmedicated depressed patients 10 females, 5 males BMI 18.8–26.4 kg/m^2	IL-6 ↑ SL Control Basal 1.50 ± 1.10 TSD 2.56 ± 1.63* Recovery 2.82 ± 1.94* Depressed Basal 1.14 ± 0.69 TSD 2.38 ± 1.87*	ND ND ND ND ND	[34] Germany
Total sleep deprivation 1 night	9 females, 1 male Bipolar disorder 36–53 years	IL-6 ? SL Basal ND TSD 3.15 ± 5.14	ND ND	[87] Italy
Total sleep deprivation 40 hours	9 females, 10 males 20–36 years BMI 18.5–24.5 kg/m^2	IL-1β ↑ PL Basal ~0.20 TSD ~0.45* IL-6 ↑ PL Basal ~1.6 TSD ~1.9*	CRP ↑ PL mg/L Basal ~0.20 TSD ~0.50*	[40] USA
Total sleep deprivation 40 hours	12 healthy males 29.1 ± 3.3 years BMI 23.4 ± 1.5 kg/m^2	IL-6 = Basal 0.60 ± 0.13 TSD 0.62 ± 0.10 Recovery 1.20 ± 0.23* TNF-α = Basal 0.88 ± 0.32TSD 1.05 ± 0.30	CRP = μg/mL Basal 1.22 ± 0.46 TSD 0.55 ± 0.13 Recovery 0.61 ± 0.14	[88] France
Total sleep deprivation 40 hours	12 healthy males 26–32 years BMI 21.9–24.9 kg/m^2	IL-6 = PL Basal ~3.5 TSD ~3.6 TNF-α ↑ PL Basal 0.66 ± 0.19 TSD 1.29 ± 0.33*	ND ND	[39] France
Total sleep deprivation 88 hours	10 healthy males 22–37 years	ND	CRP ↑ PL mg/L Basal 0.39 ± 0.13 Day 1: 0.48 ± 0.16* Day 2: 0.50 ± 0.20* Day 3: 0.65 ± 0.23* Recovery 0.66 ± 0.24*	[89] USA
Sleep restriction 5 hours time in bed (1 night)	20 males 20–22 years 71–75 kg	IL-6 ↑ PL Basal 1.89 ± 0.06 SR 3.9 ± 0.70*	ND ND	[90] Tunisia
Sleep restriction 4.2 hours time in bed (2 nights)	15 males 20–40 years BMI 20.5–24.9 kg/m^2	IL-6 = PL Basal 2.0 ± 0.0 SR 2.2 ± 0.02	ND ND	[84] Germany

Table 3: Continued.

Sleep loss condition	Subject's characteristics	Cytokines (pg/mL)	C-reactive protein	Reference
Sleep restriction 4 hours time in bed (4 days)	25 control males 25 alcoholic males	IL-6 ↑ PL Basal ~2.9 SR ~2.8 SR + Alc ~4.1 TNF-α ↑ PL Basal ~1.2 SR ~1.0 SR + Alc ~3.0	ND ND ND	[91] USA
Sleep restriction 1 hour time in bed (7 nights)	8 males 25.8 ± 0.9 years BMI 80 ± 3.7 kg/m^2 Demanding physical challenges and SR	IL-1β ↑ PL Basal 8.9 ± 2.8 SR day 7: 45.2 ± 6.3*	CRP↑ PL mg/L Basal 1.38 ± 0.89 SR Day 7: 11.38 ± 3.05*	[92] Norway
Sleep restriction 4.2 hours time in bed (10 nights)	4 females, 6 males 26–38 years BMI 21–31 kg/m^2	ND	CRP ↑ PL mg/L Basal 0.51 ± 0.20 SR 2.65 ± 1.31*	[89] USA
Sleep restriction 4 hours time in bed (10 nights)	6 females, 12 males 21–40 years BMI 20–26 kg/m^2	IL-6↑ PL Basal 1.88 ± 0.85 SR D10: 3.04 ± 2.83* Recovery 2.36 ± 1.36*	CRP = SL mg/L Basal 0.34 ± 0.27 SR Day 10: 0.69 ± 0.76	[85] USA
Sleep fragmentation OSA patients	22 females, 136 males BMI < 30 kg/m^2 28 females, 136 males BMI 30.1–34.9 kg/m^2 25 females, 107 males BMI > 35 kg/m^2	IL-6 ↑ SL 1.3 ± 0.1 1.6 ± 0.2** 2.2 ± 0.2**	CRP ↑ SL mg/L 1.8 ± 0.2 4.1 ± 0.5** 2.6 ± 0.3**	[93] Iceland
Sleep fragmentation OSA patients	148 children 6–12 years	TNF-α ↑ PL AHI ≤ 1: 3.30 ± 0.4 AHI ≥ 10: 10.02 ± 1.36*	ND	[94] Spain
Sleep fragmentation veterans	Good sleep 7 males Poor sleep 58 males	IL-1β = PL Good sleep ~1.7 Poor sleep ~3.2 IL-6 = PL Good sleep ~37.6 Poor sleep ~34.2 TNF-α = PL Good sleep ~0.8 Poor sleep ~1.2	ND ND	[95] USA

Table 3: Continued.

Sleep loss condition	Subject's characteristics	Cytokines (pg/mL)	C-reactive protein	Reference
Sleep fragmentation OSA patients	Sleep durations <6 hours, 249 males 6-7 hours, 227 males >7 hours, 135 males	IL-6 = PL <6 hours: 2.08–2.54 6-7 hours: 1.96–2.39 >7 hours: 2.00–2.59 TNF-α = PL <6 hours: 2.86–3.73 6-7 hours: 2.52–3.28 >7 hours: 2.19–3.10	CRP ↑ SL mg/L <6 hours: 1.79–2.47 6-7 hours: 1.71–2.35 >7 hours: 1.71–2.56	[96] USA

Abbreviations: AHI: apnea-hypopnea index (expressed as the number of events per hour of total sleep time); BMI: body mass index; ND: nondetermined; OSA: obstructive sleep apnea; PL: plasma levels; SL: serum levels; SR: sleep restriction; TSD: total sleep deprivation; ~: approximate values obtained from report tables; ↑: increase; =: not change; ↓: decrease; ?: without basal data; *significant differences with $P < 0.05$; **significant differences with $P < 0.01$. Mean ± standard deviation.

to proinflammatory cytokine production in peripheral blood [71]. Interestingly, studies reported differences in proinflammatory cytokine levels independent of WBC number or activity. This may be explained by considering other sources of cytokines (e.g., macrophages in adipose tissue, epithelium, and endothelium) [86], which may also be affected by sleep loss.

In addition to modifying IL-1α, IL-1β, IL-6, TNF-α, and IL-17A levels, five nights of sleep restriction are accompanied by increased heart rate; both proinflammatory cytokines and hypertension are important risk factors for development of cardiovascular disease [75, 97]. IL-17A plays a key role in sustaining tissue damage in the brain, heart, and intestine, sometimes promoting the development of autoimmune diseases [75]. Helper T cells producing IL-17A require activation by IL-6 [98]. Interestingly, IL-17A is a potent inducer of C-reactive protein (CRP) expression in hepatocytes and in coronary artery smooth muscle cells [99] (see next section). The combination of circulating cytokines with other inflammatory mediators achieves a low-grade inflammatory status induced by sleep loss.

Effect of Sleep Loss on Acute Phase Proteins. The effects of sleep loss on acute phase proteins are poorly studied. For instance, acute total sleep deprivation (one night) results in elevated high-sensitivity C-reactive protein (hsCRP) concentrations, which is a stable marker of inflammation that has been shown to be predictive of cardiovascular morbidity [89]. CRP production in the liver is stimulated by proinflammatory cytokines such as IL-6 or IL-17, which are highly expressed after sleep loss periods [75]. CRP is an important inflammatory marker because this protein lacks diurnal variations [15, 100]. In contrast, total sleep deprivation for 40 hours in young adults decreased CRP levels while increasing other inflammatory markers such as E-selectin and the intracellular adhesion molecule (ICAM)-1 [81]. Several methodological differences among the studies may contribute to the inconsistent findings for CRP (see Table 3), including the sleep deprivation period, blood sampling frequency, nutrition, and all effects and differences in subject's characteristics such as body mass index (BMI), because obesity increases proinflammatory markers [101]. In addition to voluntary sleep loss, several health conditions (e.g., pregnancy, depression) may contribute to deregulation of the immune system [102].

3.2.3. Sleep Loss and Depression. Recently, it has been suggested that one of the functions of sleep may be to regulate the neuro-immune-endocrine network [11]. In this regard, an excellent example of the interaction between the neuro-endocrine-immune network and sleep disorders is major depressive disorder, which is characterized by high levels of cortisol and TNF-α, increased NK percentages, diminished B lymphocyte counts, and no significant variations in T lymphocytes [103]; these changes are similar to the effects observed after sleep deprivation (see previous sections). In depressed patients, sleep disturbances include intermittent awakenings, prolonged sleep latency, and shortened REM sleep latency, which represent sleep fragmentation or sleep restriction (in the case of insomnia) [104, 105]. All antidepressants affect sleep architecture and quality [104], and the immune system might be altered in long-term treatment periods. For instance, depressed patients treated with selective serotonin-reuptake inhibitors for 20 weeks showed an increase in B lymphocytes [106]. The role of both major depressive disorder and sleep disturbances on the increased risk to develop metabolic disturbances is discussed in another recent review (please see [107]).

3.3. Effect of Sleep Loss on the Immune System in Animal Models

3.3.1. Effects of Sleep Loss on Cellular Immune Components. As in humans, the circadian oscillation of immune cells and molecules in rodents has been described. In mice, Ly6C^{hi} inflammatory monocyte traffic is regulated by the circadian gene Bmal1, and is higher during the resting phase and decreases during the active phase [108, 109]. Macrophages and NK contain a cell-autonomous circadian clock [110, 111]. In addition, T lymphocytes exhibit clock gene regulation,

mice immunized during the light phase show a stronger specific T lymphocyte response than those immunized during the dark phase [112]. These data suggest that a disruption of circadian rhythms might be related with changes in the WBC count after sleep loss. In rodents subjected to selective REM sleep deprivation for 24 and 240 hours, the number of T lymphocytes decreases and of B lymphocytes does not change. In the same experiment, an increase in NK percentage was observed [25]. Similarly, REM sleep deprivation for 96 hours does not promote changes in number of lymphocytes but it does increase the number of monocytes and neutrophils [33]. Controversially, REM sleep restriction promotes a decrease of leukocyte number [33]. These contradictory findings might be explained by the alteration in clock genes involved in the circadian oscillation of WBC.

3.3.2. Effects of Sleep Loss on the Molecular Inflammatory Component. Similar to humans, rodents subjected to sleep loss exhibit a proinflammatory component characterized by increase in proinflammatory cytokines, namely IL-1, IL-6, IL-17, and TNF-α as compared to control animals [32, 33]. The proinflammatory status after sleep loss may be explained, in part, because the alteration in clock genes of monocytes is associated with the upregulation of proinflammatory cytokines via NF-κB activation [76, 113]. Exposure to proinflammatory cytokines in chronic sleep restriction may promote tissue damage and subsequent loss of function; however, acute sleep deprivation may exert beneficial effects on the immune system. For instance, acute sleep deprivation is associated with a reduction in ischemia-induced IL-1β gene expression and attenuation of neuronal damage in the hippocampus. This finding may be explained by increased gene expression of IL-6 and the anti-inflammatory cytokine IL-10 after sleep deprivation [114].

4. Impact of Sleep Recovery on Sleep Loss-Induced Inflammation

Usually, the modification of cellular immune components and molecular inflammatory markers by sleep loss returns to basal levels after sleep recovery periods [34, 76]. However, depending on sleep loss time, some immune components may remain altered after sleep recovery or may even present alterations only after sleep recovery [32, 33, 100, 115]. For instance, monocyte and neutrophil numbers do not change after REM sleep deprivation in rats for 96 hours; however, after 24 hours of sleep recovery, monocyte and neutrophil numbers increase in comparison to control animals [33]. Levels of other WBC in rats decrease immediately after sleep restriction, but 24 hours of uninterrupted sleep restores the basal levels [33]. Like cellular components, molecular inflammatory mediators are altered after sleep recovery. Plasma levels of complement protein C3 were higher than controls after sleep deprivation in rats and remained elevated after sleep recovery [33]. REM sleep deprivation in rats (72 hours) increases plasma levels of IL-1, IL-6, IL-17A, and TNF-α. Proinflammatory cytokines IL-1α, IL-1β, and IL-6 return to basal levels after sleep recovery, whereas IL-17A and TNF-α remain higher than controls even after one week of normal sleep [32]. In addition, in the same study anti-inflammatory cytokines, such as IL-10, do not increase. Within the same context, in humans, increased sleepiness after sleep restriction was better reversed with a nap or with extended sleep recovery conditions (10 hours of uninterrupted sleep) [36]. In addition, other parameters associated with sleep loss were restored; for example, cortisol decreased immediately after a nap [36]. A midday nap prior to recovery sleep or an extended night of sleep can return leukocyte counts to baseline values [36]. Although long periods of sleep appear to be the solution to restore immune function, it has been reported that sleeping more than 9 hours is related with greater physical decline than midrange or short periods of sleep and also is related with increased risk of mortality associated with cardiovascular impairments [116].

5. Sleep Loss Alters the Blood-Brain Barrier

Up to this time, we have only discussed the effect of sleep loss on immune mediators at the peripheral level. Nevertheless, brain-immune system communication is very complex and it includes the direct action of proinflammatory cytokines synthesized in the brain [52, 117, 118] on neuronal systems, or the effect of peripheral cytokines on blood-brain barrier components [51]. We reported that chronic REM sleep restriction in rats induces blood-brain barrier disruption and that brief sleep recovery periods lessened these effects in several brain regions. Nevertheless, in the hippocampus hyperpermeability remained even after sleep opportunity [8]. These findings suggest that if sleep restriction increases the unselective transportation across the blood-brain barrier, proinflammatory mediators and toxic blood-borne molecules might enter the brain promoting neurochemical changes or excitotoxicity events that may explain cognitive and emotional impairments associated with sleep deficits.

6. Conclusion and Future Directions

Recent studies focus on evaluating the correlation between inflammatory markers and sleep disorders. Conditions such as obesity or infections may exacerbate the inflammatory condition contributing to systemic impairments and susceptibility to pathogens. Although sleep recovery may restore immune system alterations, when sleep loss is prolonged the proinflammatory status may remain and promote neuro-immune-endocrine axis disruption. Constant systemic inflammatory status after prolonged wakefulness may be the source of metabolic, cardiovascular, and cognitive impairments. The immune system is altered by sleep loss; however, more studies are necessary to elucidate how sleep loss promotes the release of inflammatory mediators and how these molecules act on the brain promoting local and systemic alterations that exacerbate the proinflammatory status and contribute to sleep disorders, fostering a vicious circle between inflammation and sleep disturbances (see Figure 1).

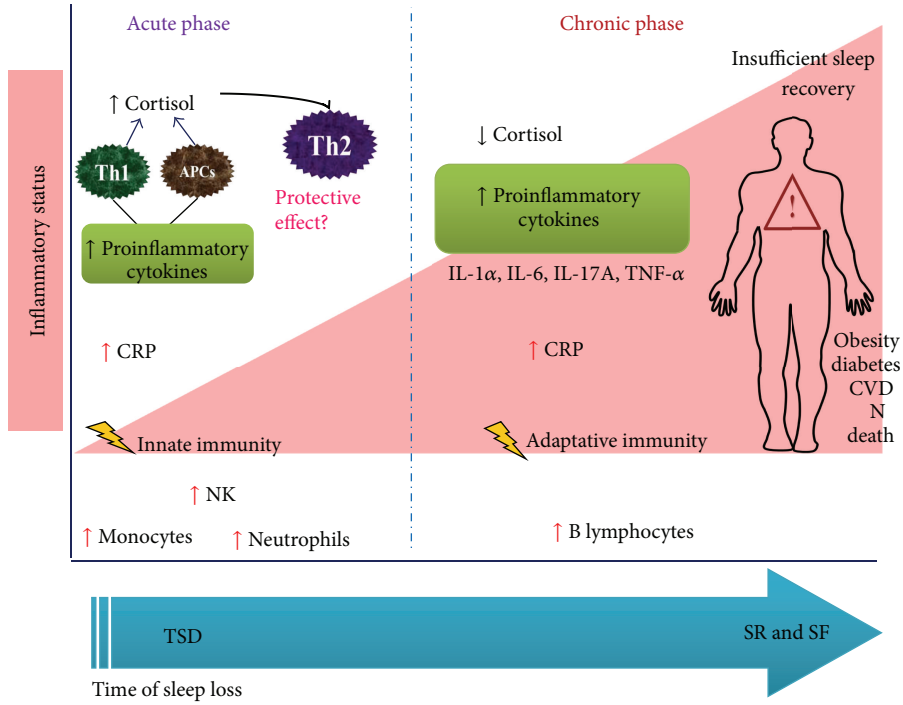

Figure 1: Sleep loss promotes a low-grade proinflammatory status. Sleep loss is characterized by an increase in circulating proinflammatory cytokines (IL-1β, IL-6, IL-17A, TNF-α) and CRP. Image shows the differential effect of sleep loss on the immune system after acute total sleep deprivation and prolonged sleep restriction and or sleep fragmentation. The acute and chronic events of sleep loss correlate with the temporal immune response (innate and adaptive). Prolonged sleep loss plus insufficient sleep recovery are considered an important risk factor to develop metabolic, cardiovascular, and neurodegenerative diseases related with the deregulation of the neuro-endocrine-immune network. Abbreviations: APCs; antigen-presenting cells; CRP, C-reactive protein; CVD, cardiovascular disease; N, neurodegenerative diseases; NK, natural killer; SR, sleep restriction; SF, sleep fragmentation; TSD, total sleep deprivation.

In the last few years, several reviews on sleep and immunity have been written. A review of some of their conclusions could be relevant. Some of them conclude that sleep modulates and is modulated by inflammation [15, 119], or that sleep deprivation impairs immune function, particularly the immune memory/humoral immune response [15, 51]. Also, some of them work with the hypothesis that sleep deprivation is a type of stress and that glucocorticoids are responsible for modifying the immune response [51]. With respect to the hypothesis that inflammation induces sleep changes, one review suggests that IL-6 is the key factor [120]; however, we need to consider that IL-6 has been proposed as a putative sleep factor and is produced by nonimmune cells [121]. We agree that there is enough evidence to conclude that inflammation modifies sleep and that sleep loss modifies circulating cytokines. If we work with the hypothesis that proinflammatory cytokines induce sleep, then we may have found a natural condition in which there is a very high level of inflammation (e.g., sepsis) and test whether sleep is changed. There are some reviews on sepsis and sleep that show that patients with sepsis present increased non-REM sleep and decreased REM sleep, with high levels of cytokines, such as TNF and IL-1β, and show an altered EEG with low-voltage, mixed-frequency waves with variable theta and delta ("septic encephalopathy") and also loss of normal circadian melatonin secretion [122]. Then, we could conclude that proinflammatory cytokines induce non-REM sleep. However, septic encephalopathy is not sleep, it is a sleep disorder, and melatonin has been successfully used in septic patients (reviewed in [122]). Thus, we come back to our hypothesis: the function of sleep is to maintain the integrity of the neuro-immune-endocrine system [11]. In this review we observe how diseases or inflammation can disrupt that integrity, and the organism will respond by modulating sleep to restore the homeostasis and also how sleep loss induces a disruption of the integrity of neuro-immuno-endocrine system causing an inadequate immune response.

Abbreviations

APCs: Antigen-presenting cells
BMI: Body mass index
CRP: C-reactive protein
EEG: Electroencephalography
ET-1: Endothelin-1
HIV: Human immunodeficiency virus
HPA: Hypothalamus-pituitary-adrenal axis
hsCRP: High-sensitivity CRP
IFN: Interferon
ICAM: Intracellular adhesion molecule

ICV: Intracerebroventricular
IL: Interleukin
MIP: Macrophage inhibitory protein
NK: Natural killer
Non-REM: Nonrapid eye movement
OSA: Obstructive sleep apnea
REM: Rapid eye movement sleep
SWS: Slow-wave sleep
T_h: T helper
TNF: Tumor necrosis factor
VGKC: Voltage-gated potassium channels
WBC: White blood cells.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contributions

Gabriela Hurtado-Alvarado, Stephanie Ariadne Castillo-García, and Beatriz Gómez-González drafted the paper. All authors reviewed the paper, and approved the final version.

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Research Article

Stimulation of TLR4 by LMW-HA Induces Metastasis in Human Papillary Thyroid Carcinoma through CXCR7

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In inflammatory sites, high molecular weight hyaluronan fragments are degraded into lower molecular weight hyaluronan fragments (LMW-HA) to regulate immune responses. However, the function of LMW-HA in PTC progression remains to be elucidated. In this study, we found that receptor of LMW-HA, TLR4, was aberrantly overexpressed in PTC tissues and cell line W3. Exposure of W3 cells to LMW-HA promoted cell proliferation and migration via TLR4. Knockdown of TLR4 has provided evidence that TLR4 is essential for LMW-HA-induced CXCR7 expression, which is responsible for LMW-HA-induced proliferation and migration of W3 cells. In tumor-bearing adult nude mice, stimulation of LMW-HA on W3 cells promotes CXCR7 expression in tumor masses ($P = 0.002$) and tumor growth ($P < 0.001$). To further confirm our findings, we investigated the clinicopathologic significance of TLR4 and CXCR7 expression using immumohistochemistry in 135 human PTC tissues and 56 normal thyroid tissue samples. Higher rates of TLR4 (53%) and CXCR7 (24%) expression were found in PTC tissues than in normal tissues. Expression of TLR4 or CXCR7 is associated with tumor size and lymph node metastasis. Therefore, LMW-HA may contribute to the development of PTC via TLR4/CXCR7 pathway, which may be a novel target for PTC immunomodulatory therapy.

1. Background

Papillary thyroid cancer (PTC) is the most prevalent thyroid cancer and represents 70 to 80% of all thyroid cancers [1]. Incidence of thyroid cancer has increased rapidly in the past 15 years the increase in incidence is almost exclusively attributable to papillary thyroid cancer [2]. Metastasis is the most important biological characteristic of PTC. That is, PTC has a tendency to spread into lymphatic channels and metastasize to regional lymph nodes at a high frequency.

It is known that inflammation plays critical roles in the development of cancers including PTC. It has been reported that T cells, B cells, and NK cells are frequently found within and surrounding primary thyroid tumor [3]. French et al. revealed that PTC patients with tumor-associated lymphocytic infiltration presented more aggressive disease when

compared with patients with concurrent thyroiditis or without lymphocytic infiltration [4], suggesting the presence of a local inflammatory response in PTC.

The extracellular matrix is important for tumor cell behavior. Hyaluronan (HA) is a polysaccharide normally expressed in the extracellular matrix of connective, neural, and epithelial tissues [5]. Under physiological conditions, HA is primarily distributed in connective tissue with many other proteins to form a large and complicated network that maintains the space between cells [5, 6]. The native, high molecular weight form (HMW-HA) is composed of repeating disaccharide units of N-acetylglucosamine and glucuronic acid (4×10^2 to 2×10^4 kDa) and is synthesized on the surface of a variety of cells. HMW-HAs are space-filling molecules that hydrate tissues they not only play a role in cell adhesion but also are antiangiogenic, anti-inflammatory, and immunosuppressive [7]. HMW-HA can be degraded into lower molecular weight fragments (LMW-HA) by hyaluronidases, whose expression is elevated during the process of inflammatory responses, tumor development, and tissue injury [3, 8]. Recent study shows that LMW-HA, not the native HMW-HA, can initiate inflammatory responses in dendritic cells in skin transplant rejection [9]. It has been reported that there are three receptors of LMW-HA, TLR2, TLR4 and CD44. Binding of LMW-HA to these receptors could activate immune cells and promote the production of different cytokines by macrophages, activated dendritic cells, and T cells [10–13].

Tumor cells have also been shown to produce hyaluronidases, which lead to HA degradation in the tumor surrounding environment [14]. Voelcker et al. suggested that LMW-HA in melanoma might promote tumor invasiveness by inducing MMP and cytokine expression, partly in a TLR4-dependent manner [15], providing new insights into the relationship between cancer and innate immunity. Moreover, Bourguignon et al. suggested that LMW-HA played an important role in CD44-TLR-associated AFAP-110-actin interaction and MyD88-NF-κB signaling, which was required for tumor cell behaviors [16]. Besides, LMW-HA inhibits colorectal carcinoma growth by decreasing tumor cell proliferation and stimulating immune response [17]. Bohm et al. suggested that strong stromal HA staining intensity was related to the progression and unfavorable outcome in differentiated thyroid carcinoma (DTC) patients including PTC [18]. However, little else is known about the function of LMW-HA in PTC development.

Key molecules such as chemokines/chemokine receptors not only attract leukocytes to local inflammatory sites but also directly enhance the survival, proliferation, and migration of tumor cells. The chemokine CXCL12 (also called stromal-derived factor-1) is an important chemokine that binds CXCR4/CXCR7, playing important roles in promoting tumor cell proliferation and migration [19].

In this study, we investigated the roles of LMW-HA in the progression of PTC. We found that TLR4 was aberrantly overexpressed in PTC. Moreover, stimulation of LMW-HA induced CXCR7 expression in PTC cells via TLR4 signaling to promote the proliferation and migration of PTC cells. Furthermore, tumor-bearing mice and clinicopathology were used to verify that the LMW-HA/TLR4/CXCR7 pathway may be critical during the development of PTC, indicating LMW-HA as a possible novel immunomodulatory therapy target for PTC treatment.

2. Material and Methods

2.1. Cell Lines. Human PTC cell line K1 was purchased from the American Type Culture Collection. Cell lines, W3, and TPC1 were kind gifts from Dr. Robert Gagel (MD Anderson Cancer Center, University of Texas, USA). All cells were cultured at 37°C and 5% CO_2. K1 cells were maintained in DMEM : Ham's F12 : MCDB 105 (2 : 1 : 1) (Invitrogen) and supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 100 μg/mL streptomycin (Invitrogen) and 100 U/mL penicillin (Invitrogen). W3 cells were maintained in RPMI 1640 (Invitrogen) with 10% FBS (Invitrogen) and 100 μg/mL streptomycin (Invitrogen) and 100 U/mL penicillin (Invitrogen). TPC1 cells were maintained in DMEM (Invitrogen) with 10% FBS (Invitrogen) and 100 μg/mL streptomycin (Invitrogen) and 100 U/mL penicillin (Invitrogen).

2.2. Animals and Tumor Model. Female nude mice (6–8 week) were purchased from the Shanghai Laboratory Animal Center at the Chinese Academy of Sciences and housed in a specific pathogen-free facility at the Shanghai Jiao Tong University School of Medicine. All animal procedures were approved by the Animal Welfare & Ethics Committee of Shanghai Jiao Tong University School of Medicine. Tumors were xenografted onto the left flank of mice through a subcutaneous injection of 6×10^6 W3 cells in 50 μL of phosphate buffered saline (PBS). Mice were intratumorally injected with LMW-HA (4,900 Da, JIANGSU HAIHUA BIOTECH CO, China, 400 μg/kg) or the same volume of DMSO every other day. Tumors were measured with a caliper every fourth day and tumor volumes were calculated using the formula (length $\times$ width^2)/2. When maximum diameters of tumors reached about 1.0 cm, mice were euthanized. Tumors were removed and weighed. CXCR7 expression in tumor tissues was analyzed by immunohistochemistry.

2.3. Patients and Specimens. This study was approved by the Medical Ethics Committee of The First Affiliated Hospital of Bengbu Medical College and all works were conducted in accordance with the Declaration of Helsinki. All participants gave informed written consent before participating in this study. PTC samples were collected from 135 patients undergoing curative-intent surgery at the Department of Surgery, The First Affiliated Hospital of Bengbu Medical College between 2001 and 2011. There were also 56 normal thyroid tissue samples adjacent to papillary thyroid carcinoma (used as controls). The histologic sections were reviewed by two expert pathologists to verify the histologic diagnosis. None of the patients had received any preoperative treatment. Tumors were staged according to the American Joint Committee on Cancer (AJCC) pathologic tumor-node-metastasis (TNM) classification.

2.4. Western Blot Analysis. PTC cell lines were lysed with RIPA Lysis Buffer (Beyotime, China) supplemented with protease inhibitor Cocktail (AppliChem, Germany). Protein concentration in the postnuclear lysates was measured by BCA Protein Assay Kit (Beyotime, China) and equal amounts of protein lysates (60 μg) were loaded on 10% SDS-PAGE. Gels were transferred to nitrocellulose using iBlot Dry Blotting System (Invitrogen, USA). Filters were blocked with 5% dry skimmed milk and blotted with the specific primary antibodies: mouse monoclonal antibody to TLR4 (Abcam, England), TLR2 (eBioscience, USA), or CD44 (eBioscience, USA). Blots were then incubated with the appropriate HRP-conjugated secondary antibody (Beyotime, China), and signals were detected by the WestPico chemiluminescence system (Pierce). Filters were stripped for 10 min with ReBlot Plus Strong Antibody Stripping Solution (Millipore).

2.5. Flow Cytometric Assay. For *in vitro* studies of CXCR7 and CXCR4 expression, all cell lines were cultured in medium containing 2% FBS. After 12 h, W3 cells were treated with LMW-HA for 24 h. Then cells were trypsinized and incubated for 1 h with a monoclonal anti-human CXCR7 antibody (R&D systems, USA) or CXCR4 antibody (BD Biosciences, USA) and analyzed using a flow cytometry (BD Aria).

2.6. Cell Transfection. siRNA sequences (TLR4: 5′-GAGCCGCUGGUGUAUCUUU-3′, TLR4-KD; CXCR7: 5′-CCGUUCCCUUCUCCAUUAU-3′, CXCR7-KD; Scrambled siRNA: 5′-AGGACTGAGTGTACCGTCT-3′, Scram) were designed into shRNA and inserted into pGPU6/GFP/Neo vector (GenePharma, Shanghai, China) under U6 promoter. Cells resistant to G418 (800 μg/mL) were selected and expanded for further study. The depletion of endogenous TLR4 or CXCR7 by the shRNA was confirmed by immunoblot.

2.7. Cell Proliferation Assay. W3 cells were cultured in 96-well plates at an initial density of 2,000 cells per well, in 100 μL of 1% FBS-medium with or without addition of LMW-HA (100 ng/mL) for indicated times. Cell proliferation was determined using a WST-1 Kit (Beyotime, China). Each experimental condition was sampled in triplicate and the experiments were repeated three times.

2.8. Apoptosis Assay. W3 cells were incubated in 1% FBS-medium with or without addition of LMW-HA for 24 hours and incubated for 30 min at room temperature with 0.5 mg/mL propidium iodide (PI, eBioscience, USA) and annexin V-FITC (eBioscience, USA). Then cells were analyzed with flow cytometry. Each experiment was repeated three times.

2.9. Migration Experiments. W3 cells were resuspended in 1% FBS-medium at 5×10^5 cells/mL and seeded into the upper chambers of Transwell inserts (Millipore). 1% FBS-medium was added to the lower chambers, with or without addition of CXCL12 (100 ng/mL). After incubation with LMW-HA, the nonmigrated cells were removed from the upper surface of the filters, and the migrated cells, adherent to the lower surface, were counted (Ten high-power fields/well). Each experiment was repeated three times.

2.10. Immunohistochemistry Assay. Sections were subjected to routine deparaffination and rehydration. Antigen retrieval was achieved by microwaving in 0.01 mol/L citrate buffer for 10 min and then cooled for 30 min. The endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide in methanol for 20 min and nonspecific binding was blocked with 5% bovine serum albumin in PBS at room temperature. After three PBS washes, the specimens were incubated overnight at 4°C with murine anti-human TLR4 and CXCR7 monoclonal antibodies. After incubation with rat anti-mouse-IgG2b-horseradish peroxidase, signal was developed with 3, 30-diaminobenzidine tetrahydrochloride in Tris-HCl buffer (pH 7.6) containing 0.02% hydrogen peroxide. The sections were then counterstained with hematoxylin and mounted. Negative controls were performed by replacing the primary antibody with nonspecific IgG at the same concentration.

2.11. Interpretation and Evaluation of Immunohistochemical Results. Immunostaining was independently examined by two clinical pathologists who were unaware of the patient outcome. For each sample, five high-power fields (100×) were randomly selected. Staining intensity and percentage of positive tumor cells were assessed. The extent of the staining was categorized into five semiquantitative classes based on the percentages of positive tumor cells: 0 (<5% positive cells), 1 (6–25% positive cells), 2 (26–50% positive cells), 3 (51–75% positive cells), and 4 (>75% positive cells). The intensity of cytoplasmic and membrane staining was also determined semiquantitatively on a scale of 0–3 as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). A consensus score was assigned for each section after discussion and careful review of all slides by the two pathologists. Multiplication of the intensity and the percentage scores gave rise to the final staining score: negative (0), + (1–4), ++ (5–8), and +++ (9–12). For statistical analysis, tumors having a final staining score of negative or +, which showed a weak or moderate/strong immunoreactivity, were grouped into a low expression group and were compared to tumors with scores of ++ or +++ as the high expression group.

2.12. Statistical Analysis. Differences were evaluated using the Statistical Package for Social Science software (version 16.0, SPSS Inc., Chicago, IL). The association of staining intensity with clinicopathologic patterns was assessed with the Chi square test and two-sided Fisher's exact test to determine the significance of the difference between the covariates. All measurement data are presented as mean ± SEM. Statistical significance was evaluated by one-way ANOVA, followed by the least significant difference (LSD) test. P values <0.05 were considered to be statistically significant.

3. Results

3.1. TLR4 Is Highly Expressed in PTC Tissues and Cell Lines. It has been shown that lymphocytic infiltration presented in or around PTC tissues, which mediated local inflammatory responses and affected the progression of PTC [4]. In sites of

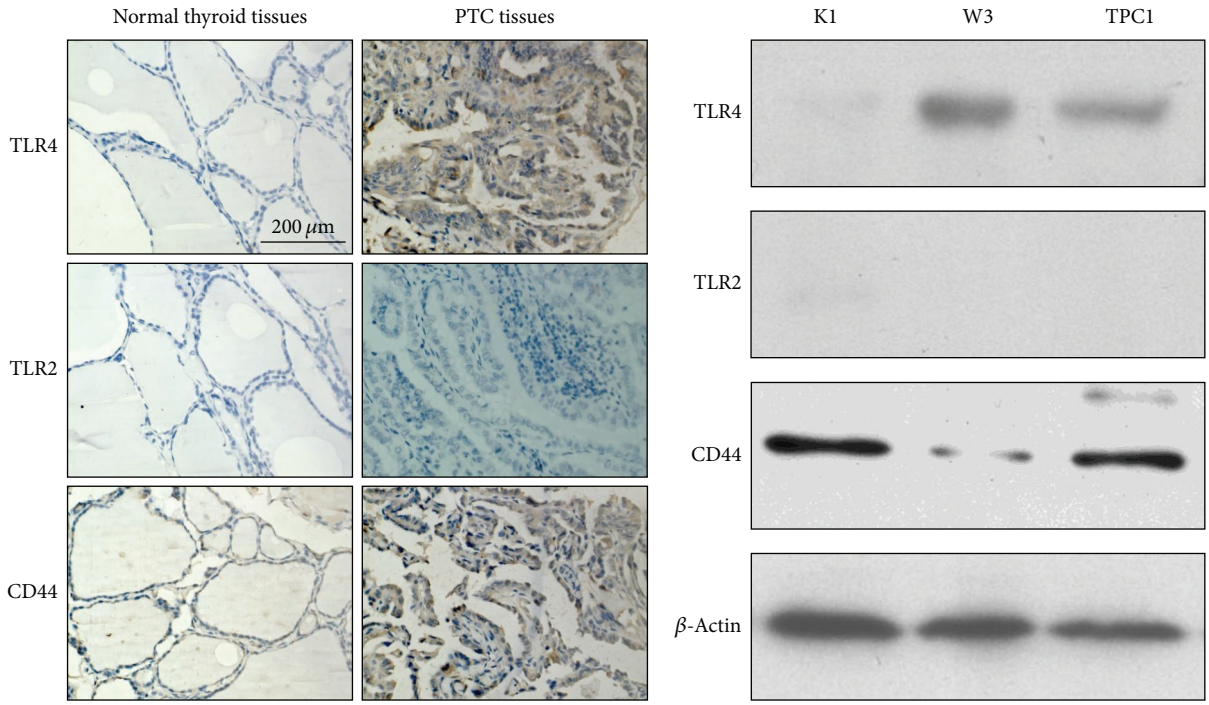

Figure 1: Increased TLR4 expression in PTC tissues and cell line W3. (a) Representative examples of IHC staining analyses of TLR4, TLR2, and CD44 in human normal thyroid tissues and PTC tissues (original magnification 400×). (b) Immunoblot analysis of the expression of TLR4, TLR2, and CD44 on 3 human PTC cell lines. Representative results are shown.

inflammation, HMW-HA may be degraded into LMW-HA, which in return activates immune responses [3, 11]. It has been reported that strong stromal HA staining intensity is related to progression and unfavourable outcome in thyroid carcinoma patients [18]. To investigate whether LMW-HA played a role in the development of PTC, we assessed the expression levels of three LMW-HA receptors, TLR2, TLR4, and CD44, in PTC tissues and three different human PTC cell lines (K1, W3, and TPC1). Immunohistochemistry analysis showed that TLR4 was highly expressed in PTC tissues compared to normal thyroid tissues (Figure 1(a), Table 1, $P < 0.001$); TLR2 was virtually undetectable (<10% of cells) in both normal thyroid tissues and PTC tissues; CD44 was expressed in most of normal thyroid tissues (51 of 56) and PTC tissues (128 of 135) (data not shown). These data demonstrate that TLR4 is aberrantly overexpressed in PTC, suggesting that LMW-HA/TLR4 may participate in the development of PTC. At the same time, western blot analysis indicated that TLR4 was highly expressed on W3 cells, moderately expressed on TPC1 cells, and low or negatively expressed on K1 cells; CD44 was weakly expressed on W3 cells and strongly expressed on K1 and TPC1 cells; while TLR2 expression could not be detected on three of these cell lines (Figure 1(b)). As CD44 expression did not show difference between normal thyroid tissues and PTC tissues, $TLR4^{high}CD44^{low}$ PTC cell line, W3 cells were chosen for this study thereafter to exclude the possible effects of LMW-HA/CD44 signal on tumor progression.

3.2. LMW-HA Promotes Proliferation and Migration of W3 Cells via TLR4. Then we treated W3 cells with LMW-HA and determined the effects of LMW-HA on W3 cell apoptosis, proliferation, and migration. Stimulation of LMW-HA significantly enhanced the proliferation of W3 cells (Figure 2(a)) but did not induce apoptosis of W3 cells (Figure 2(b)). Moreover, LMW-HA significantly promoted the migration of W3 cells in the presence of CXCL12 (Figure 2(c)). Next we investigated if LMW-HA promotes proliferation and migration of W3 cells via the receptor, TLR4. When TLR4 expression was knocked down by shRNA (Figure 2(d)), LMW-HA induced proliferation and migration of W3 cells were mostly abolished (Figures 2(e) and 2(f)). In addition, we further stimulated TLR4 negative K1 cells with LMW-HA, and the data showed that LMW-HA did not promote the proliferation and migration of K1 cells (see Supplemental Figures (a) and (b) available online at http://dx.doi.org/10.1155/2013/712561). Collectively, these data suggest that LMW-HA promotes the proliferation and migration of W3 cells through activating TLR4 signal pathway.

3.3. LMW-HA Elevates CXCR7 Expression to Promote Proliferation and Migration of W3 Cells. To determine how LMW-HA promotes proliferation and migration of W3 cells, downstream molecules of TLR4 signal pathway, CXCR4 and CXCR7 expression were examined in W3 cells treated with or without LMW-HA. Neither CXCR4 nor CXCR7 was

Table 1: Correlation of TLR4 and CXCR7 expression with clinicopathologic features in PTC.

Clinicopathologic parameters	Case no.	TLR4 expression		P value	CXCR7 expression		P value
		Low	High		Low	High	
Total cases	135	64	71	53%	102	33	24%
Age							
≤60	55	27	28	$P = 0.745$	38	19	$P = 0.080$
>60	80	37	43		64	15	
Tissue type							
Normal tissue	56	55	1	$P = 0.000$	55	1	$P = 0.000$
Carcinoma	135	64	71		102	33	
Sex							
Male	65	33	32	$P = 0.451$	48	17	$P = 0.656$
Female	70	31	39		54	16	
Tumor size							
≤5 cm	58	39	19	$P = 0.000$	53	5	$P = 0.000$
>5 cm	77	25	52		49	28	
TNM stage							
I	4	4	0	$P = 0.000$	4	0	$P = 0.005$
II	65	39	26		57	8	
III	38	7	31		24	14	
IV	28	14	14		17	11	
Histologic grade							
I	8	7	1	$P = 0.000$	8	0	$P = 0.002$
II	109	56	53		86	23	
III	18	1	17		8	10	
Lymph nodemetastasis							
Negative	76	50	26	$P = 0.000$	70	6	$P = 0.000$
Positive	59	14	45		32	27	
Distant metastasis							
Negative	108	56	52	$P = 0.039$	89	19	$P = 0.000$
Positive	27	8	19		13	14	

expressed in W3 cells, whereas exposing W3 cells to LMW-HA induced significant CXCR7 expression (Figure 3(a)). In contrast to CXCR7 expression alterations, LMW-HA had no effect on CXCR4 expression in W3 cells (Figure 3(b)). To investigate the role of TLR4 in LMW-HA-mediated CXCR7 expression, we knocked down TLR4 in W3 cells. Then LMW-HA-mediated CXCR7 expression was totally inhibited (Figure 3(c)). Next we assessed whether upregulation of CXCR7 expression was responsible for LMW-HA induced proliferation and migration of W3 cells. We found that knockdown of CXCR7 (Figure 3(d)) indeed blocked the proliferation and migration alterations in W3 cells induced by LMW-HA (Figures 3(e) and 3(f)). Moreover, LMW-HA also did not upregulate CXCR7 expression in TLR4 negative K1 cells. Taken together, these findings suggest that LMW-HA/TLR4-induced CXCR7 expression significantly promotes PTC cell proliferation and migration.

3.4. Stimulation of W3 Cells by LMW-HA Promotes Tumor Growth in Adult Nude Mice. To substantiate the effects of LMW-HA on tumor growth, female BALB/c nude mice were subcutaneously injected with W3 cells and treated with or without LMW-HA. The volumes of the tumor masses formed in LMW-HA treatment groups were larger than those of tumors from the control treatment groups (Figure 4(a)). To determine the effect of LMW-HA/TLR4/CXCR7 pathway on tumor growth *in vivo*, TLR4 or CXCR7 was inhibited with shRNA in W3 cells. Tumor growth was substantially inhibited in TLR4-KD and CXCR7-KD group in contrast to scrambled siRNA group in the presence of LMW-HA (Figure 4(b)), suggesting that LMW-HA may promote growth of W3-derived PTC model tumors *in vivo* through TLR4/CXCR7 pathway. Then immunohistochemistry analysis demonstrated that CXCR7 expression was significantly higher in tumor masses treated with LMW-HA in contrast to that of control treatment groups (Figure 4(c); Table 2, $P = 0.002$). Knockdown of

Table 2: CXCR7 expression in W3 cell transplanted tumor tissues of nude mice.

Group	n	CXCR7 expression		P value
		Low	High	
LMW-HA	6	2	4	$P = 0.002$
DMSO	6	6	0	

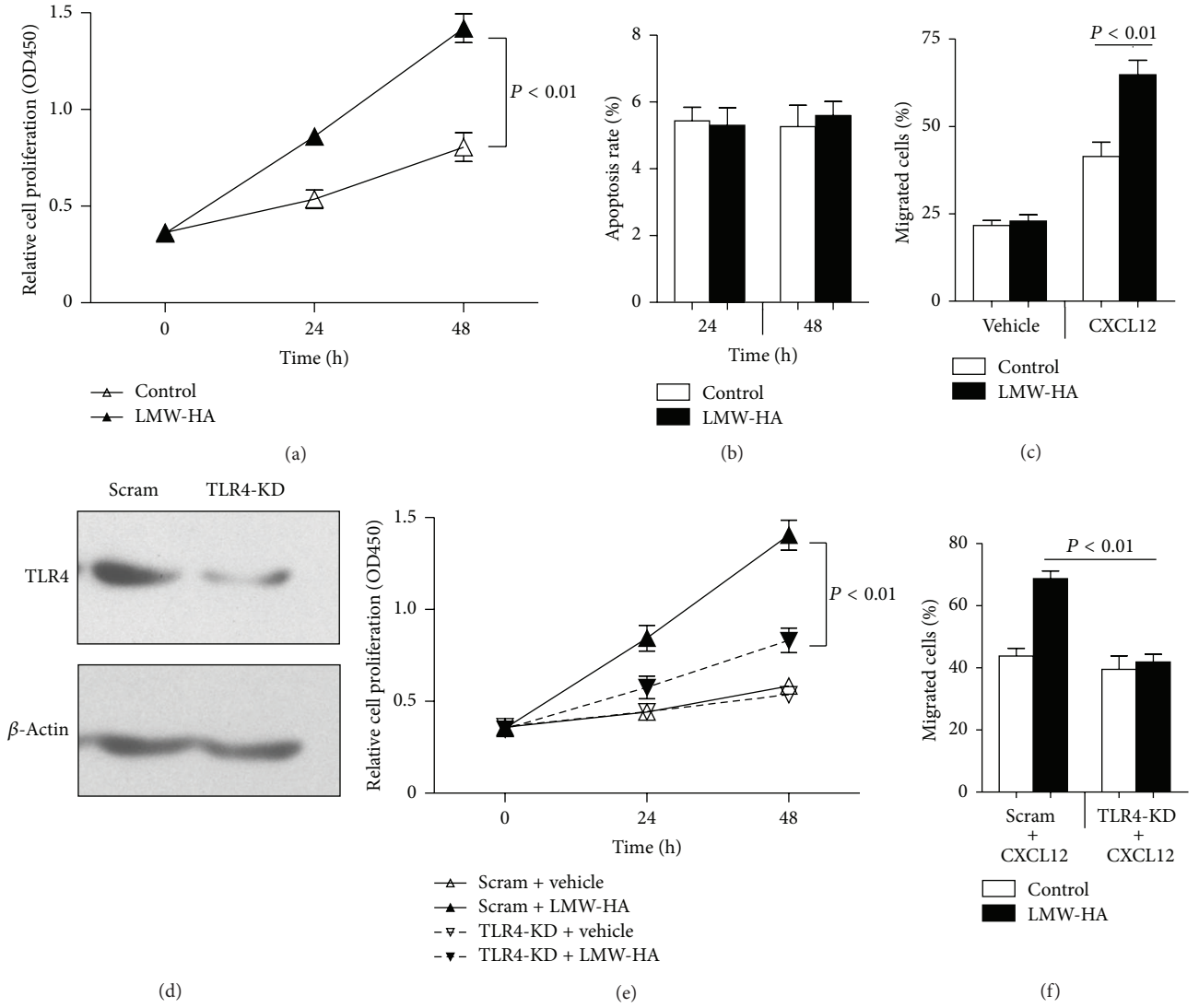

Figure 2: LMW-HA promotes W3 cell proliferation and migration via TLR4. (a) W3 cells were seeded into 96-well plates (2,000 cells/well) and treated with or without LMW-HA. Cell proliferation was analyzed with WST-1 Kit. Data are mean ± SEM for three independent experiments. (b) W3 cells were treated with or without LMW-HA for 24 hours and stained with annexin V and PI. Data are mean ± SEM for three independent experiments. (c) W3 cells were seeded into the upper chambers of transwell inserts treated with or without LMW-HA and in the presence or absence of CXCL12 in the lower chambers. Migrated cells were determined. Data are mean ± SEM for three independent experiments. (d) W3 cells were transfected with scrambled shRNA (Scram) or TLR4 shRNA-expressing constructs (TLR4-KD) and subjected to immunoblot analysis. (e) Scram-W3 cells and TLR4-KD W3 cells were seeded into 96 well plates and treated with or without LMW-HA (100 μg/mL) for 24 h; cell proliferation was analyzed. Data are mean ± SEM for three independent experiments. (f) Scram-W3 cell and TLR4-KD W3 cell migration to CXCL12 treated with or without LMW-HA was determined. Data are mean ± SEM for three independent experiments.

TLR4 inhibited LMW-HA-induced expression of CXCR7 in tumor masses, indicating that CXCR7 was induced by LMW-HA in tumor tissue through TLR4 and might play important roles in tumorigenicity.

3.5. Expression of TLR4 or CXCR7 Is Associated with Tumor Size and Lymph Node Metastasis. To further determine whether LMW-HA/TLR4/CXCR7 pathway plays a role in PTC progression, we investigated the clinicopathologic significance of TLR4 and CXCR7 expression using immunohistochemistry in human PTC tissues. TLR4 and CXCR7 exhibited mostly cytoplasmic and plasmalemmal staining in carcinoma tissues (Figures 1(a) and 5). Normal tissue adjacent to tumor cells showed negative or occasionally weak staining that was mostly cytoplasmic (Figures 1(a) and 5). The differences in expression of the two molecules between carcinoma tissues and normal thyroid tissues were all found to be statistically significant (Table 1; TLR4, $P < 0.001$; CXCR7, $P < 0.001$). As shown in Table 1, tumor size tended to be larger in cases with high rather than low expression of TLR4 ($P < 0.001$) and CXCR7 ($P < 0.001$). There is a statistically significant correlation between TNM stage and TLR4 expression

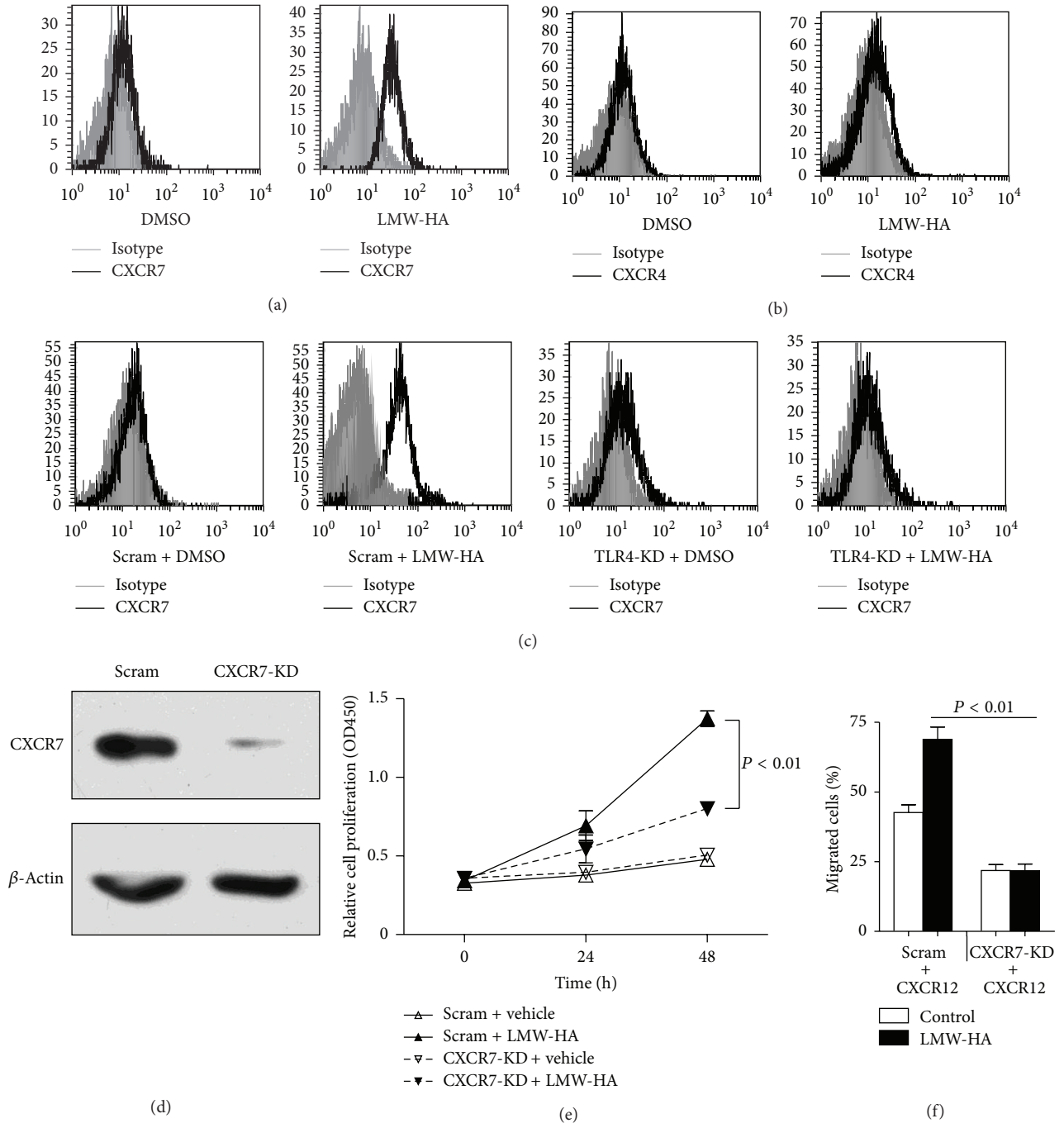

Figure 3: LMW-HA upregulates CXCR7 to promote W3 cell proliferation and migration. ((a)-(b)) W3 cells were incubated with LMW-HA (100 μg/mL) for 24 h; representative flow cytometric analysis of CXCR7 (a) or CXCR4 (b) expression was shown. (c) Scram-W3 cells and TLR4-KD W3 cells were treated with or without LMW-HA (100 μg/mL) for 24 h; representative flow cytometric analysis of CXCR7 expression was shown. (d) W3 cells were transfected with scrambled shRNA (Scram) or CXCR7 shRNA-expressing constructs (CXCR7-KD), and subjected to immunoblot analysis. (e) Scram-W3 cells and CXCR7-KD W3 cells were seeded into 96 well plates (2,000 cells/well) and treated with or without LMW-HA. Cell proliferation was analyzed. Data are mean ± SEM for three independent experiments. (f) Scram-W3 cell and CXCR7-KD W3 cell migration to CXCL12 treated with or without LMW-HA was determined. Data are mean ± SEM for three independent experiments.

($P < 0.001$) or CXCR7 expression ($P = 0.005$). The increased expression is significantly associated with advanced histological grade (TLR4, $P = 0.001$; CXCR7, $P = 0.002$). At the same time, the incidence of lymph node metastasis tended to be higher in patients with PTC with high rather than low expression of TLR4 ($P < 0.001$) or CXCR7 ($P < 0.001$). In addition, the incidence of distant metastasis tended to be higher in patients with PTC with high rather than low expression of TLR4 ($P = 0.039$) or CXCR7 ($P < 0.001$). There were no statistically significant differences in these molecules with

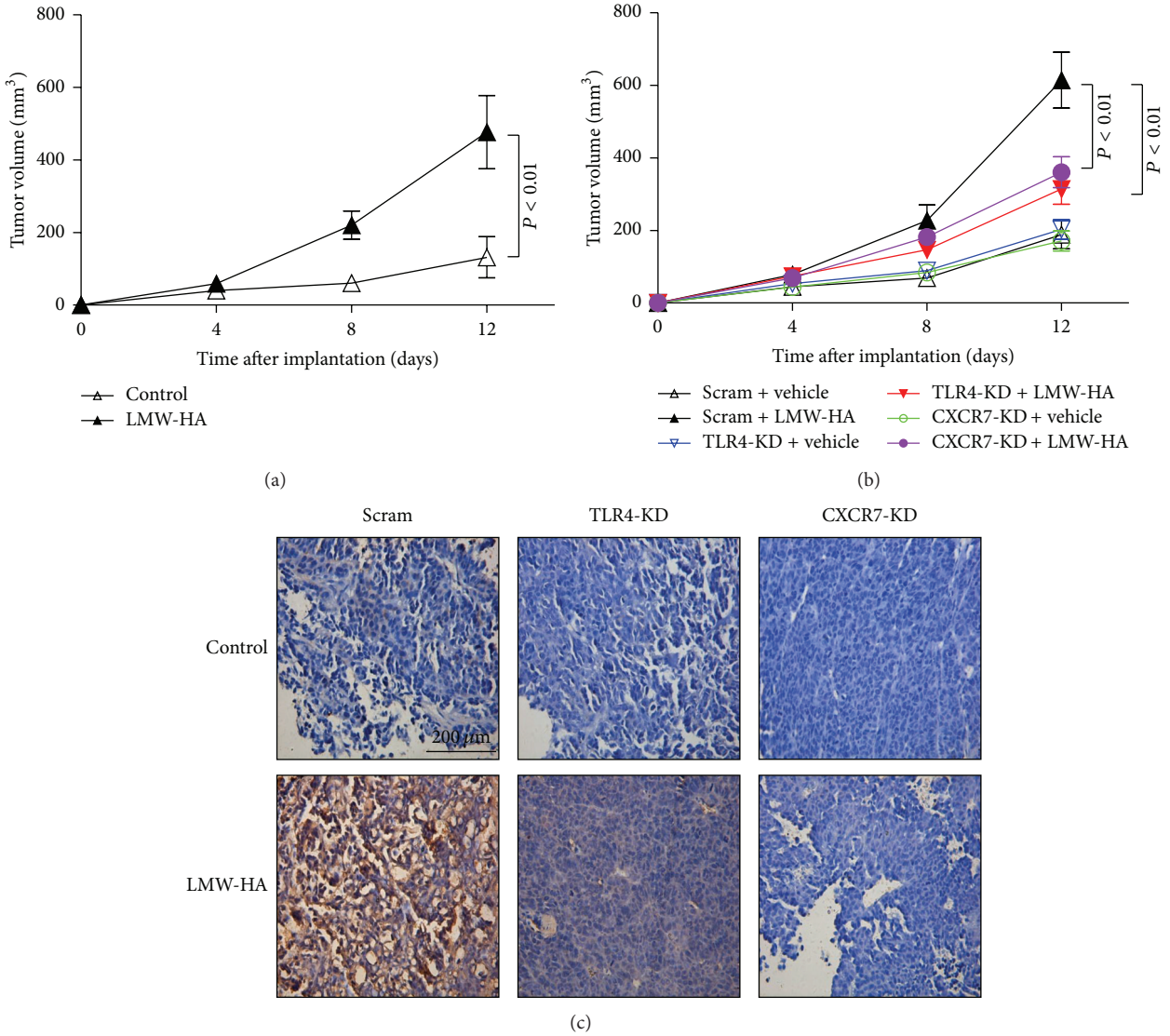

Figure 4: LMW-HA enhances the tumorigenicity of W3 cell line via TLR4/CXCR7 pathway. (a) W3 cells (6×10^6 cell/mouse) were injected subcutaneously into the flanks of nude mice, and mice were intratumorally injected with LMW-HA (400 μg/kg) or the same volume of DMSO every other day. Tumors were measured with a caliper every fourth day. When tumor maximum diameter reached about 1.0 cm, mice were euthanized and tumors were removed and weighed. The volumes of the tumor masses formed in LMW-HA treatment groups and control treatment groups were determined. (b) Scram-W3 cells, TLR4-KD W3 cells, and CXCR7-KD W3 cells were injected subcutaneously into the flanks of nude mice, and mice were intratumorally injected with LMW-HA (400 μg/kg) or the same volume of DMSO every other day. Tumor volumes were determined and shown. (c) CXCR7 expression of tumors was analyzed by immunohistochemistry (original magnification 400×). These results were representative of three independent experiments.

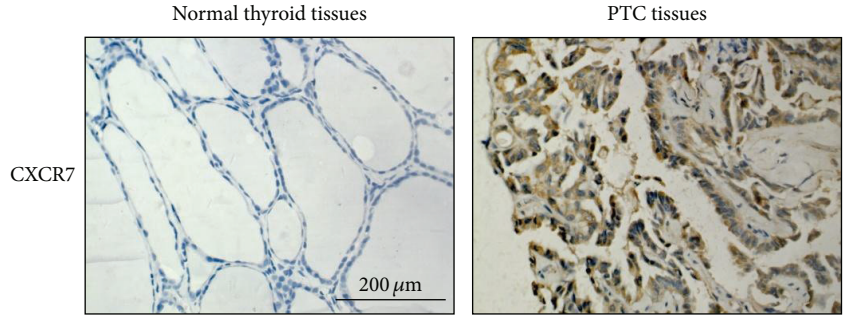

Figure 5: Increased expression of CXCR7 in PTC tissues. Representative examples of immunohistochemical staining of CXCR7 in normal thyroid tissues and PTC tissues (original magnification 400×). Representative results are shown.

regard to patient age and sex. All these data indicate that expression of TLR4 or CXCR7 is associated with PTC tumor size and lymph node metastasis.

4. Discussion

Metastases, rather than primary tumors, are responsible for most cancer deaths. This process requires tumor cells to acquire the ability of proliferation, antiapoptosis, migration, and invasion. The presence of central neck lymph node metastases in PTC is known as an independent risk factor for recurrence. Our data showed that LMW-HA induced CXCR7 upregulation in PTC cells through TLR4 signaling, which promoted PTC cell line W3 proliferation and migration. Likewise, LMW-HA could also promote W3 cell proliferation in nude mice. Furthermore, higher rates of TLR4 and CXCR7 expression were found in human PTC tissues than in normal thyroid tissues, indicating that expression of these two molecules is associated with increased carcinoma growth and metastasis potential in human PTC.

In sites of inflammation or tissue injury, HA, ubiquitous in the extracellular matrix, is broken down into LMW-HA that has been reported to activate immunocompetent cells. For instance, it induces inflammatory chemokine and cytokine expression in macrophages [20]. Black et al. demonstrated a novel CD44 and MyD88 independent pathway for HA fragments to activate macrophage production of interferon-β via TLR4-TRIF-TBK1-IRF3 [21]. Stimulation of TLRs by LMW-HA induces self-defense mechanisms in vaginal epithelium [22]. LMW-HA increases the self-defense of skin epithelium by induction of β-defensin 2 via TLR2 and TLR4 [13]. LMW-HA and HMGB1 act as innate immune cytokine-like signals with the potential to modulate chondrocyte differentiation and function in OA progression via MyD88-dependent TLR2/TLR4 signaling [23]. Recently, LMW-HA has been shown to be associated with tumor invasiveness and metastasis [24]. Our data in this study have unraveled the crucial mechanisms underlying the promoting effect of inflammation-derived-LMW-HA signaling on the metastatic potential of PTC cells. A marked increase of CXCR7 expression was induced in a TLR4 positive PTC cell line W3, in response to LMW-HA. Knockdown of TLR4 in W3 cells has provided evidence that TLR4 is essential for LMW-HA-induced CXCR7 expression. Simultaneously, we established LMW-HA-W3 tumor-bearing mice model to further determine the function of the LMW-HA/TLR4/CXCR7 pathway in PTC.

CD44 is a primary cell-surface HA receptor. Binding of HA to CD44 plays roles in cell adhesion, immune responses, and tumor development. Although it has been reported that HA-CD44 signaling promotes the progression of several cancers, such as breast cancer [25], colorectal carcinoma [17], fibrosarcoma [26], and glioblastoma multiforme [27], in this study we found that CD44 was overexpressed in both normal tissue and PTC tissue and overexpression of CD44 was not relevant to progression of PTC. Further *in vitro* study may be needed to investigate the effect of CD44 on the proliferation and migration of PTC cell lines.

TLR4 expressed on tumor cells has been found to contribute to tumor progression by promoting tumor cell proliferation, apoptosis resistance, and tumor evasion from immune attack [28, 29]. LPS was released from the damaged cells or from bacteria in tumor tissues. Once LPS binds to TLR4, two signaling pathways are activated: a MyD88-dependent pathway and a MyD88-independent pathway [30, 31]. Based on studies using macrophages, these pathways are responsible for the expression of proinflammatory cytokines [32–34]. Clinical and experimental studies indicate that TLR4 plays a significant role in connecting inflammation and cancer invasion and progression, but the exact mechanism is still not clear. The chemokine CXCL12/SDF-1 and its receptor, CXCR4, have been implicated in invasion, survival, and proliferation of carcinoma cells [35]. Recently, CXCR7 was identified as a second receptor for CXCL12 [36, 37]. Though, some results have indicated that CXCR7 functions as a decoy receptor [38], growing evidence suggested that CXCR7 significantly increases cell proliferation and elevates cellular adhesion property in some conditions [36, 38–41]. We observed that CXCR7 induced by LMW-HA could promote metastasis of PTC cell line W3. However, LMW-HA had no effect on CXCR4 expression. In animal model, differences in CXCR7 expression in tumor masses between the two groups were statistically significant. In addition, the incidence of lymph node metastasis and distant metastasis tended to be higher in patients with PTC with high rather than low expression of TLR4 or CXCR7. Conclusively, the LMW-HA/TLR4/CXCR7 pathway is involved in the development of PTC, suggesting that LMW-HA/TLR4 signaling may be an effective immunomodulatory therapeutic target in PTC.

5. Conclusion

In conclusion, we demonstrated that LMW-HA could promote the development of PTC. After binding to TLR4, LMW-HA activated TLR4 signal pathway to promote PTC cell proliferation and migration through upregulation of CXCR7 expression. We also suggested that aberrant expression of TLR4 and CXCR7 in PTC was associated with poor progression of PTC. Therefore, taking the LMW-HA/TLR4/CXCR7 pathway as the potential immunomodulatory therapy target may be a promising approach for PTC treatment.

Conflict of Interests

All authors have no conflict of interests.

Acknowledgments

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Research Article

Modulation of LPS-Induced CD4+ T-Cell Activation and Apoptosis by Antioxidants in Untreated Asymptomatic HIV Infected Participants: An *In Vitro* Study

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Persistent immune activation characterises HIV infection and is associated with depletion of CD4+ T-cells and increased risk of disease progression. Early loss of gut mucosal integrity results in the translocation of microbial products such as lipopolysaccharide (LPS) into the systemic circulation. This is an important source of on-going immune stimulation. The purpose of this study was to determine levels of CD4+ T-cell activation (%CD25 expression) and apoptosis (% annexin V/7-AAD) in asymptomatic, untreated HIV infection at baseline and after stimulation with LPS and incubation with or without vitamin C and N-acetylcysteine. LPS induced a significant ($P < 0.03$) increase in %CD25 expression, annexin V, and 7-AAD in HIV positive individuals. NAC in combination with vitamin C, significantly ($P = 0.0018$) reduced activation and early apoptosis of CD4+ T-cells to a greater degree than with either antioxidant alone. Certain combinations of antioxidants could be important in reducing the harmful effects of chronic immune activation and thereby limit CD4+ T-cell depletion. Importantly, we showed that CD4+ T-cells of the HIV positive group responded better to a combination of the antioxidants at this stage than those of the controls. Therefore, appropriate intervention at this asymptomatic stage could rescue the cells before repetitive activation results in the death of CD4+ T-cells.

1. Introduction

HIV infection is characterized by chronic immune activation and inflammatory cytokine production [1, 2]. The consistent activation of CD4 and CD8 T-cells is associated with depletion of CD4 T-cells and increased risk of disease progression to AIDS [3]. Furthermore, markers of immune activation have been shown to be stronger predictors of progression to AIDS than either the CD4 counts or viral loads [4–6]. In particular, increased T-cell activation has been associated with AIDS suggesting that activated T-cells are susceptible to apoptosis [7]. In addition, elevated levels of CD38, a marker of immune activation on CD4 and CD8 T-cell, predict a rapid decrease of CD4 T-cells and a shorter survival rate, independent of HIV viral loads [8, 9].

The significant depletion of memory-type CD4 T-cells lining the gastrointestinal tract (GIT) mucosa in early HIV infection results in the breakdown of the mucosa and on-going translocation of microbial products such as lipopolysaccharide (LPS) across the epithelial surface [1, 10]. LPS induces activation of innate immune cells such as monocytes and dendritic cells resulting in increased oxidative stress; depletion of antioxidant defence mechanisms and an increased susceptibility to apoptosis [1, 11]. In addition, CD4 T-lymphocytes have been shown to express-toll-like receptor-4 (TLR4), which is a receptor for LPS [12, 13]. Triggering of TLR4 activates various signalling pathways such as mitogen-activated protein kinases (MAPKs), p38, and JNK, which induce activation of transcription factor nuclear factor of kappa B (NF-κB) and subsequent production of

proinflammatory cytokines, chemokines, antimicrobial peptides, and other defence molecules such as ROS [13]. The proinflammatory cytokines such as TNF-α, are capable of activating innate immune cells to produce more ROS. ROS and proinflammatory cytokines in turn induce activation of both the extrinsic and intrinsic pathways of apoptosis. The role of *in vitro* stimulation with lipopolysaccharide (LPS) on T-cell activation in HIV has been explored only minimally [14, 15]. The first study to date in HIV used CD38 and HLA-DR as the activation markers which were conducted on HIV positive patients who were on antiretroviral treatment [15]. Few studies have investigated the inhibitory effects by antioxidants on immune activation and apoptosis in asymptomatic, untreated HIV infection. In the current study, we investigated the effects of vitamin C and NAC on LPS-induced upregulation of interleukin receptor-2 receptor alpha chain, (CD25), as a marker of LPS-induced activation of CD4+T-cells after overnight incubation in untreated HIV infection.

In this study, therefore, levels of immune activation and apoptosis were measured before and after stimulation with LPS and incubation with selected antioxidants (vitamin C and NAC) in untreated HIV positive individuals. These levels were compared to a control group. We developed an assay that demonstrates the response of CD4+ T-cells to LPS-induced stimulation and, further, showed the inhibitory effect of antioxidants in this process.

2. Materials and Methods

2.1. Study Population and Design. In this cross-sectional study, twenty untreated, asymptomatic HIV positive individuals and 20 controls (32 females and 8 males) were sourced from a single HIV testing and prevention primary health clinic in Crossroads, Cape Town, South Africa. The median age of the participants was 32 years (range 22–42). There was no significant difference in age between the two groups. The HIV positive group had a significantly ($P = 0.0003$) lower CD4 count compared to the control group. The patients' demographics are summarised in Table 1. Informed consent was taken from all the participating subjects. Inclusion criteria for the study participants were 21 years or older, individuals with HIV infection and CD4 counts >200; not on antiretrovirals (ARVs) or any other chronic medication or antioxidant supplements. Exclusion criteria included patients with tuberculosis (TB) or other coinfections and those receiving antiretroviral therapy, anti-TB treatment or other antibiotic treatment, antioxidant supplementation, mineral and vitamins supplements, aspirin, or any other drug, for example, anti-inflammatory.

Ethics approval was obtained from both the clinical site, University of Cape Town: REC: REF: 417/2006 and laboratory site, University of Stellenbosch HREC N07/09/197.

2.2. Reagents. Flow-check Fluorospheres, Flow-set Fluorospheres, CD4-PE, CD4-APC, CD25-PE, and annexin V-FITC/7AAD-PE kit were obtained from Beckman Coulter, Miami Florida Inc. (USA). FC 500 cytometer with two lasers, from Beckman Coulter, Miami, Florida, USA, FL, was used to acquire the data. L-ascorbic acid stock powder or vitamin C ($C_6H_8O_6$; molecular weight 176.12 g/mol; 25 g powder) and N-acetyl-L-cysteine stock powder ($C_5H_9NO_3S$; 25 g powder) were purchased from Sigma-Aldrich (South Africa).

2.3. Sample Preparation. Blood was drawn into two 10 mL tubes with Heparin, one 5 mL tube with EDTA (for viral load) and one 5 mL tube with citrate (for D-dimers). Samples were then couriered from the clinic to the laboratory within two hours of collection.

Heparinized whole blood samples were incubated with antioxidants for 20 min then stimulated with LPS, incubated overnight, and analysed on flow cytometer. Briefly, 100 μL of blood was added into the labelled tubes and 30 μL of vitamin C (10 mM) or 20 μL of NAC (5 μM) vortexed gently and incubated for 20 minutes. An additional tube was prepared with the "cocktail" of both antioxidants. After 20-minute incubation of the samples at 37°C with 5% carbon dioxide (CO_2), 20 μL of LPS (2 μg/mL) was added. The dosages (2 μg/mL LPS, 10 mM vitamin C, and 5 μM NAC) used in this study were chosen for these experiments after a rigorous optimization study on the effects of temperature, time, and concentration on LPS-induced whole blood activation and antioxidant intervention in asymptomatic untreated HIV infection previously done in our lab (data not shown); these doses were chosen (data not shown). The samples were incubated overnight and analysed on flow cytometer.

For each sample, 100 μL of blood was added to appropriately labelled Beckman's flow tubes and 10 μL of monoclonal antibody mix was added. The sample was vortexed gently and incubated at room temperature for 15 minutes in the dark after which 500 μL of fluorescein-activated cell sorting (FACS) lysing solution was added. The sample was vortexed gently and incubated for 15 minutes at room temperature. After incubation, 250 μL of ice cold staining buffer and 250 μL of binding buffer were added. The sample was spun at 300 g for 5 minutes after which 750 μL of supernatant was removed. The pellets were resuspended, 200 μL of staining buffer and and 200 μL of binding buffers were added and analysed on flow cytometer.

2.4. Flow Cytometry Analysis. An FC 500 flow cytometer (Beckman Coulter, Miami, FL, USA) with two lasers, five fluorescence channels, and CXP analysis software were used in this study. Alignment of the lasers was performed with a mixture of Flow-check and Flow-check beads. The appropriate voltages were determined and standardized with a mixture of Flow-set and Flow-set beads. Full matrix colour compensation was done using FITC, PE, APC, and PerCP/PC5 stained whole blood cells prepared using the lyse and wash method. A panel was created for test analysis using the cytometer settings established with flow-set and full matrix colour compensation. CXP and FCS Express V3 software programs were used to analyse the flow cytometry data.

Table 1: Demographics characteristics of both the HIV positive and control groups.

Parameter	HIV positive group ($n = 20$)	Controls ($n = 20$)	P values
Male : female	5 : 15	3 : 17	
Median age (yrs)	31.8 (27–35)	30.3 (22–35)	0.43
Range	21–51	21–48	
Median CD4 cells/mm^3	411 (265–634)	753 (564–870)	0.0012**
Median viral load (copies/mL)	45705 (2174–157294)	ND	
Log viral load	4.0 (3.1–5.4)		

All the values in columns are median (interquartile range) of HIV positive ($n = 20$) individuals and controls ($n = 20$). **Significant at $P = 0.05$.

2.5. Data Acquisition and Analysis for Apoptosis. For apoptosis, plot quadrants were set using unstained cells for every sample such that the negative annexin V cells and 7-AAD negative population lay in the first decade of the Y and X axis. A sequential gating strategy, by first gating on lymphocytes for CD4+ T-cells and then gating on CD4 T-cells for annexin V versus 7-AAD,was performed. This was in order to detect Annexin V + 7-AAD negative cells (apoptotic cells), Annexin V negative 7-AAD positive cells (dead cells), and Annexin V positive 7-AAD positive (secondary apoptotic or necrotic) CD4 T-cells as shown in Figure 1. A total of 300,000 events were acquired in order to analyse a minimum of 2000 CD4+ T-cells. CXP and FCS express V3 software programmes were used to analyse flow cytometry data.

2.6. Markers of Disease and Immune Activation. CD4 T-cell counts were determined by staining whole blood with Becton Dickinson (BD) MultiTest CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent in BD TruCount tubes according to the manufacturer's instructions and analysed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). HIV-1 RNA quantifications were performed using 1.0 mL of plasma with the use of the Nuclisens Easy Q HIV-1 v.1.2 kit (BioMerieux Inc., Boxtel, the Netherlands). CD38 expression on CD8+ T-cells (CD38/8) was determined by flow cytometry. Whole blood samples were incubated with the monoclonal antibodies: CD8-Per-CP; and CD38-APC; CD3-FITC (BD Biosciences, San Jose, CA, USA) and analysed on a BD FACSCalibur instrument using BD Cell Quest Pro (Version 2) software. Lymphocytes were gated on forward versus side scatter, CD3, and CD8 expression.

2.7. D-Dimers. D-dimer, a marker of fibrinogen breakdown and clot formation, thus an indirect marker of inflammation, was determined by spectrophotometry using the IL-D-dimer method. This is an automated immunoassay for quantitative determination of D-dimers in plasma. Plasma from sodium citrate blood samples was mixed with latex reagent and buffer all supplied by Beckman Coulter (Miami, FL, USA) and agglutination, measured as decrease in absorbance, was read at 405 nm using ACL TOP from Beckman Coulter (Miami, FL, USA).

2.8. Statistical Analysis. The data was analysed using the Graphpad Prism version 5 statistical analysis software. Comparisons between the groups (HIV+ and HIV−) were done. Analysis of variance (ANOVA) was used to determine whether the means of the two groups (HIV+ and HIV−) differed significantly. Mann-Whitney nonparametric test and spearman's correlation were applied. Results were reported as medians with interquartile ranges. A 5% or lower significance level was used to determine significant findings ($P \leq 0.05$).

3. Results

3.1. Demographics of Study Population. The participants' demographics are summarized in Table 1. The group included 20 HIV positive and 20 controls most of whom were females (Fisher's test $P = 0.36$). There was no significant ($P > 0.05$) difference between the two groups in terms of age. Both groups had similar mean ages: 31 years for HIV positive group and 30 years for the control group. The HIV positive group had a significantly ($P = 0.0012$) lower CD4 count compared to the control group. The HIV positive group had a well-maintained CD4 count averaging 464 cells/mm^3 (median-411 cell/mm^3) and was clinically well. Median viral load was 45705 copies/mL. The control group had a high CD4 count with an average of 746 cells/mm^3 and was also clinically well.

3.2. %CD25 Expression in the HIV Positive and Control Groups. The %CD25 expression before and after stimulation with LPS and incubation with vitamin C and NAC of the forty study participants is shown in Table 2 and illustrated in Figure 2. Baseline (unstimulated) levels of activation were not significantly different between the two groups ($P = 0.40$), however, after stimulation, the HIV positive group showed statistically significant increase in activation ($P = 0.03$) when compared to the controls, which was not significant ($P = 0.16$). A significant difference was noted with the incubation of LPS and vitamin C alone and NAC alone: in the control group. Optimal levels of inhibition of activation in the HIV group were achieved with the combination of NAC + vitamin C ($P = 0.0018$).

3.3. The % Annexin V/7-AAD Staining for Early and Late Apoptosis between the HIV Positive and Control Groups. The % Annexin V/7-AAD staining before and after stimulation with LPS and overnight incubation with vitamin C and NAC is summarised in Tables 3 and 4, and Figures 3 and 4. For early apoptosis, at baseline, the levels of annexin V+/7AAD-staining were not significantly ($P > 0.05$) different between the two groups; however, a significant difference was noted

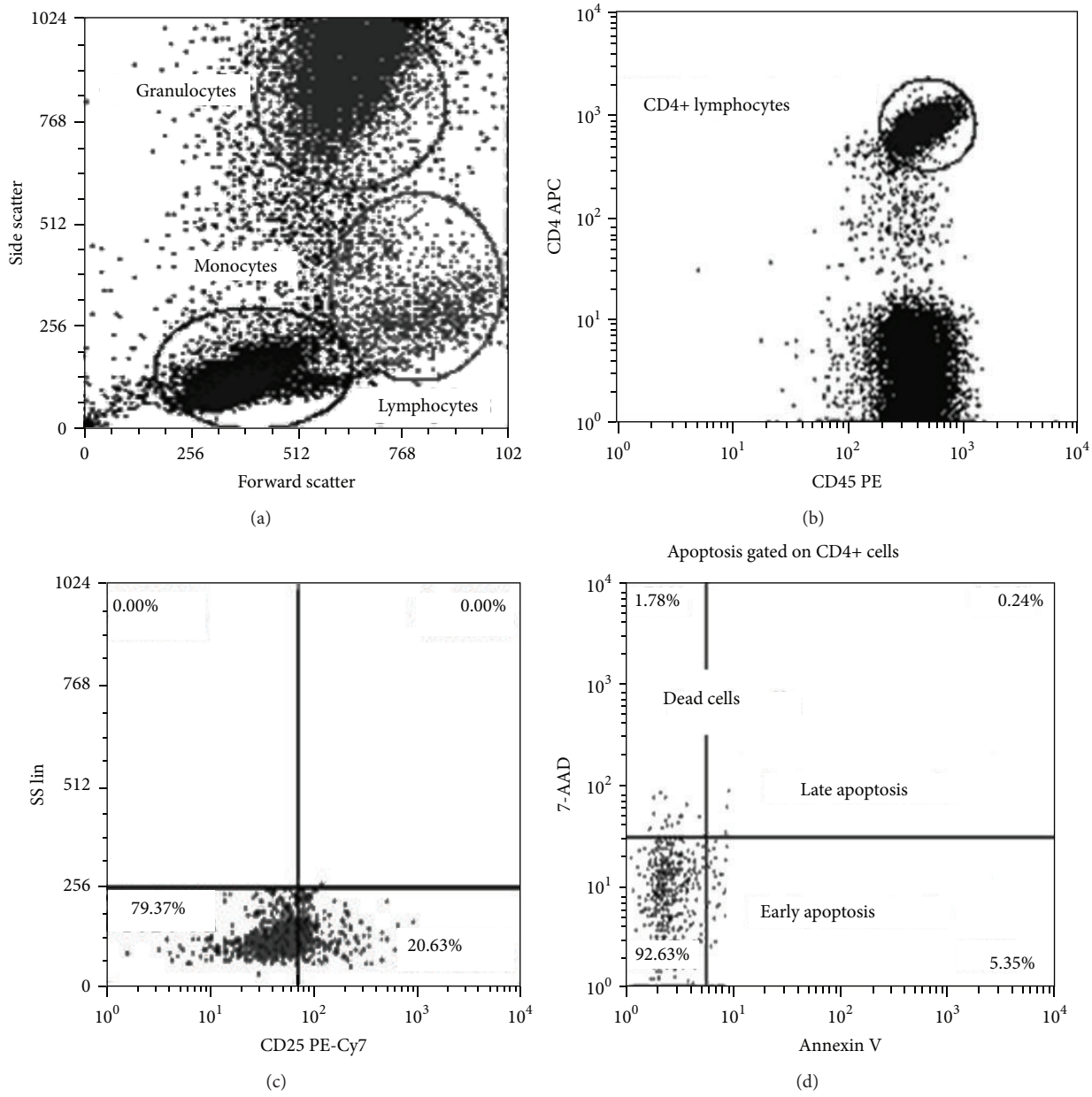

Figure 1: Gating strategy for activation (CD25) and apoptosis (annexin V/7-AAD). Plots (a) (side scatter versus forward scatter), (b) (bright CD4+ cells gated from the CD45+ leukocytes), (c) (CD25+ cells gated from the CD4 gate) and (d) (early, late apoptosis, and dead cells gated from CD4+ cells gate) show dot plots of whole blood used to set the quadrants.

Table 2: The %CD25 expression before and after overnight stimulation with LPS and incubation with vitamin C and/or NAC.

%CD25	HIV+ group	Controls	*P* value between HIV and control	*P* value for LPS activation and inhibition by antioxidants in HIV group	*P* value for control
Unstimulated	7.9 (7.1–14.4)	10.2 (7.8–14.4)	0.4047		
LPS stimulated	12.5 (10.3–17.6)	11.6 (9.6–15.2)	0.6823	0.0337**	0.16
LPS + Vit C	15.2 (11.7–17.6)	8.6 (7.2–11.9)	0.0003**	0.4	0.01**
LPS + NAC	9.0 (7.1–14.2)	9.9 (6.9–13.1)	0.9033	0.0416**	0.07
LPS + cocktail	6.0 (4.2–13.1)	9.1 (7.8–14.6)	0.0501	0.0018**	0.22

All the values are median (interquartile range) percentages of cells expressing CD25 of the HIV positive and control groups, unstimulated and stimulated with LPS and incubated overnight. ** Medians significant at $P < 0.05$.

Table 3: The % annexin V+/7-AAD− staining of the HIV positive and the control groups.

% Annexin V	HIV positive group	Control	*P* value (HIV and controls)	*P* value for LPS activation and inhibition by antioxidants in HIV+ group	*P*-value for controls
Unstimulated	1.6 (0.9–4)	1.9 (1.2–2.3)	0.49		
LPS-activated cells	3.3 (1.6–5.6)	2.5 (2.0–3.2)	0.17	0.11	0.007**
LPS + VIT C	3.6 (1.9–4.2)	2.6 (2.1–3.4)	0.26	0.84	0.44
LPS + NAC	3.4 (1.2–4.3)	2.6 (2.2–3.4)	0.55	0.54	0.66
LPS + CKTL	1.8 (1.0–3.7)	1.9 (1.5–2.4)	0.96	0.02**	0.008**

All the values are median (interquartile range) percentages of cells staining with annexin V/7-AAD− of the HIV positive and control groups, unstimulated and stimulated with LPS and incubated overnight. *Medians are marginally significant at $P = 0.05$. **Medians significant at $P < 0.05$.

Table 4: The % annexin V+/7-AAD+ staining of the HIV positive and HIV negative groups.

%7-AAD	HIV positive group	Control	*P* value between HIV and control	*P* value for LPS activation and inhibition by antioxidants in HIV group	*P* values for the controls
Baseline	0.7 (0.4–2.2)	0.4 (0.3–0.5)	0.02**		
LPS-activated cells	1.9 (0.7–3.2)	0.6 (0.4–0.7)	0.0029**	0.10	0.14
LPS + VIT C	1.2 (0.7–2.7)	0.7 (0.4–1.0)	0.0048**	0.89	0.27
LPS + NAC	2.4 (1.0–3.4)	0.6 (0.4–0.8)	0.0003**	0.64	0.79
LPS + CKTL	1.8 (1.3–2.1)	0.7 (0.6–1.2)	0.0001***	0.76	0.56

The values are median (interquartile range) percentages of cells staining for both annexin V and 7-AAD of the HIV positive and control groups, unstimulated and stimulated with LPS and incubated overnight. **Medians significant at $P < 0.05$. ***Medians significant at $P \leq 0.0001$.

after stimulation with LPS ($P = 0.007$) in the control group. There was a significant difference after incubation with LPS and a combination of NAC and vitamin C in both groups. Importantly, the combination of vitamin C and NAC significantly ($P < 0.0001$) reduced the annexin V+/7-AAD- staining cells back to its unstimulated levels. NAC and vitamin C in combination significantly ($P = 0.007$, $P = 0.002$) decreased the staining of annexin V+/7-AAD- cells. For late apoptosis, there was no significant difference ($P > 0.05$) before stimulation with LPS and after stimulation with LPS and incubation with the antioxidants individually or in combination, meaning that these antioxidants are effective in limiting early apoptosis. This could help in retaining their functionality and protecting them from early death.

3.4. Other Markers of Disease in HIV Infection. Table 5 shows the values of other markers of disease and immune activation in the cohort. When compared with the controls as expected, the HIV positive individuals had significantly ($P < 0.05$) lower CD4 counts. D-dimers, an indirect (marker of fibrinogen breakdown and clot formation) marker of inflammation, was significantly ($P < 0.05$) higher in HIV positive patients than in controls.

There was a strong inverse correlation between CD4 count and viral load ($r = -0.62$; $P = 0.03$) and negative correlation between CD4 count and %CD38/CD8+ ($r = -0.48$; $P = 0.05$). However, there was no correlation between CD4 counts and D-dimers.

4. Discussion

Persistent immune activation characterises HIV infection and is associated with the depletion of CD4+ T-cells, increased risk of disease progression, and higher mortality. The breakdown of gut mucosal integrity in early HIV infection results in translocation of microbial products such as lipopolysaccharide (LPS) into the systemic circulation. This is an important source of on-going immune stimulation. In this study, therefore, we developed an assay to determine the ability of CD4+ T-cells in HIV to be activated by LPS *in vitro* and further to be inhibited by selected antioxidants.

LPS induced a significant increase in CD25 (activation marker) expression in HIV infection when compared to that of the controls ($P = 0.68$). Thus, at this stage of the infection, with relatively well-maintained CD4 counts and no clinical symptoms, CD4 T-cells in HIV infection retain the ability to be activated, which was significantly reduced by NAC and a combination of NAC and vitamin C. After incubation with antioxidants and stimulation with LPS, interestingly, the HIV positive group showed good responses to NAC alone and a "cocktail" of NAC and vitamin C, with CD25 levels returning to below baseline values, however, a similar effect could only be demonstrated in the control group with vitamin C alone. The combination of vitamin C and NAC was required to achieve optimal inhibition of the LPS-induced-activation.

LPS induces activation of innate immune cells such as monocytes and dendritic cells resulting in increased production of proinflammatory cytokines consequently inducing

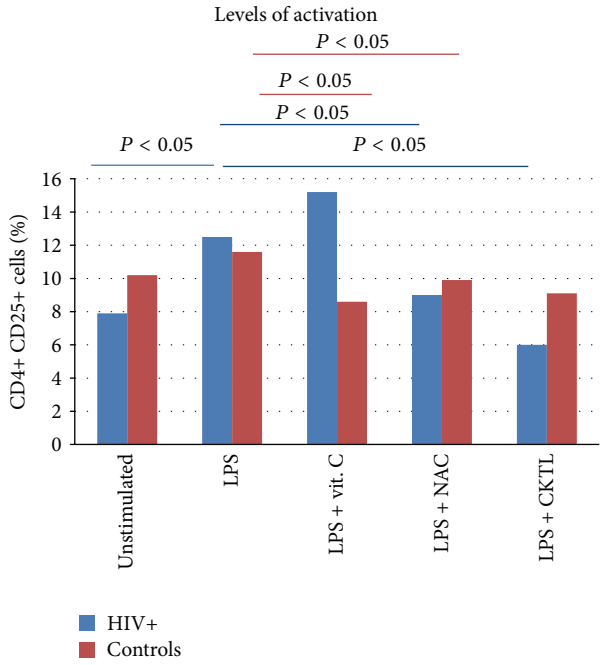

Figure 2: The figure shows the median %CD25 expression on CD4+ T cells under different experimental conditions for both HIV positive and control groups. LPS induced a significant increase in CD25 expression in HIV infection ($P = 0.03$) and this increase was similar to that of the controls ($P = 0.68$). Red and black lines at the top of the bars indicate significance.

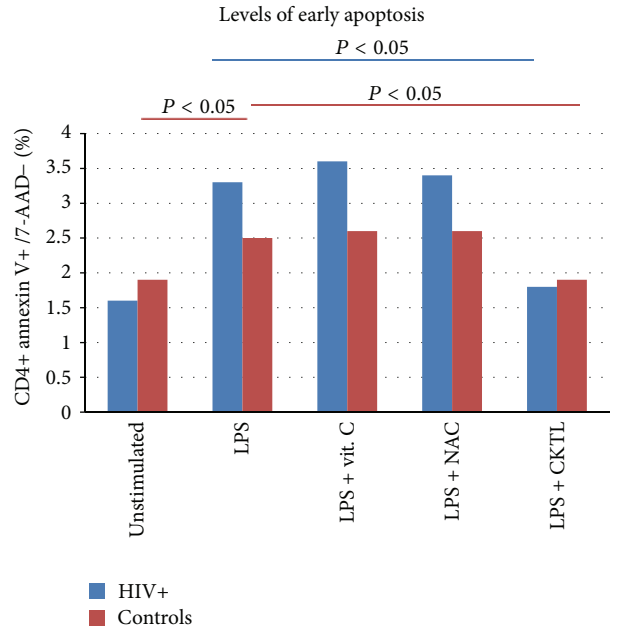

Figure 3: The figure shows the median % annexin V staining on CD4+ T-cells under different experimental conditions for both HIV positive and control groups. LPS induced a significant increase in annexin V staining in the controls ($P = 0.007$) and this increase was significantly reduced in both HIV positive and controls ($P = 0.02$; $P = 0.008$, resp.) by a combination of vitamin C and NAC. Red and blue lines at the top of the bars indicate significance.

oxidative stress. This leads to depletion of antioxidant defence mechanisms and an increased susceptibility to apoptosis. CD25 is a relatively late marker of activation [16]. In the study by Tincati et al. on the effects of *in vitro* LPS stimulation on T-cells in patients on HAART, significantly higher CD4+ and CD8+ expressing HLA-DR and CD38+ expressing cells were detected in low and intermediate responders compared to the HIV negative group confirming a sustained immune activation in HIV infection [15]. The current study confirms Tincati et al. findings of increased LPS-induced immune activation as measured by CD25 expression in HIV infection when compared to controls. Furthermore, there was a significantly ($P < 0.05$) increased immune activation expressed as CD38 in the HIV positive group when compared to the controls.

Annexin V staining was also significantly increased after stimulation with LPS in HIV positive and control groups but was more pronounced in the HIV positive group. NAC alone and in combination with vitamin C significantly reduced early apoptosis of CD4+ T-cells to a greater degree than vitamin C alone. Moreover, LPS induced a significant increase in 7-AAD staining in HIV infection, which was significantly reduced by the antioxidants either alone or in combination.

This study demonstrates that LPS was capable of inducing CD4 T-cell activation and apoptosis *in vitro* as indicated by increased CD25, annexin V, and 7-AAD, which was ameliorated by the combination of antioxidants. In addition, NAC alone significantly reduced LPS-induced activation and apoptosis of CD4+ T-cells in HIV infection. Early work

Table 5: Other markers of disease in HIV infection in both the HIV positive and control groups.

	HIV positive group	Control group	*P* value
D-dimers (mg/L)	0.23 (0.2-0.3)	0.21 (0.20–0.26)	<0.0001**
CD38/8 (%)	27.6 (17.5–44.0)	11.6 (7.3–15.8)	<0.0001**

The table shows the D-dimers and %CD38/8 in these groups expressed as median (interquartile range). ** indicates that the medians were significantly different at $P = 0.05$. CD38/8 was significantly different ($P < 0.0001$).

demonstrated that NAC administration to HIV positive individuals was able to slow down CD4 decline in HIV infection [17]. NAC and glutathione have been shown to completely block activation-induced death and associated DNA fragmentation in T-cell hybridomas, therefore implicating redox regulation in the processes [18]. In addition, NAC has been shown to directly scavenge free radicals by decreasing hypochlorous acid produced by neutrophils [19, 20]. Cell studies have indicated that NAC enhances intracellular killing of bacteria by protecting the neutrophils and macrophages from free radicals generated during phagocytosis [21]. In support of the current study findings, NAC has been shown previously to inhibit LPS-mediated activation; however, this work was performed in rats kupffer cells [14]. The effect of NAC on T-cell activation and apoptosis particularly in the context of HIV infection is not well documented and therefore the findings of this study may be of value in the future management of persons living with HIV.

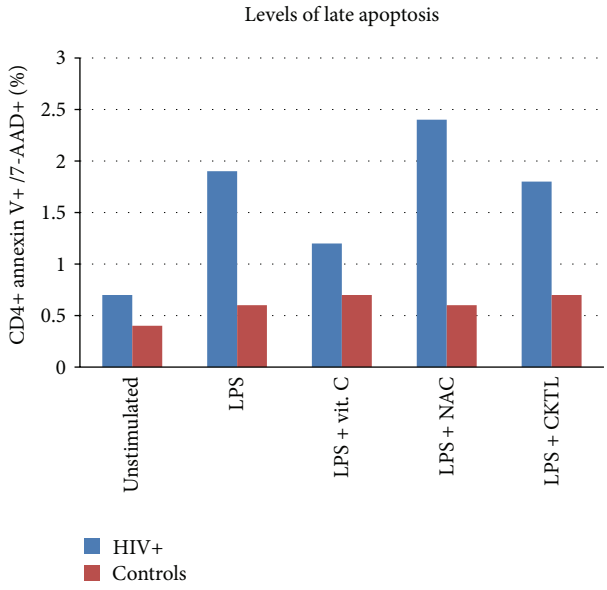

Figure 4: The figure shows the median % annexin V+/7-AAD+ staining on CD4+ T-cells under different experimental conditions for both HIV positive and control groups. LPS did not induce a significant increase in annexin V+/7-AAD+ staining in both groups ($P > 0.05$).

An important finding of this study was that vitamin C had no effect on LPS-induced activation when used alone in HIV, but when used in combination with NAC (cocktail), it showed a significant reduction in CD4+ T-cell activation levels. Previous studies have reported clinical improvement in AIDS patients who willingly consumed high doses (500 mg, 800 mg and 1 800 mg) of ascorbic acid [22–25]. In addition vitamin C has been shown to inhibit NF-κB activation via multiple stimuli including IL-1 and TNF in endothelial cell lines ECV30VS and in primary HUVECS [26]. Several studies have shown that vitamin C inhibited T-cell pathways of apoptosis which includes the upregulation of the antiapoptotic B-cell lymphoma-2 (Bcl-2) protein expression levels [27–29]. Although vitamin C exhibits strong antioxidant properties, it has been demonstrated *in vitro* that it can also act as a prooxidant in the presence of free transition metals [30, 31]. In this setting, it generates hydroxyl radicals in a fenton-like reaction. This could explain the cause of activation of CD4+ T-cells when vitamin C was used alone in the current study. In support of this, Bergman et al. demonstrated a 39% increase in apoptotic cells when cells were incubated with 0.2 mg/mL vitamin C for 24 hrs [32]. Previous data on clinical trials using vitamin C have been conflicting. Some authors have suggested that supplementation with vitamin C is toxic [33, 34]. In this study, only low concentrations (10 mM) of vitamin C were utilized. Previous work in our laboratory (results not shown) had demonstrated that higher concentrations were toxic and able to cause activation and even death of the cells. Thus, at higher doses, vitamin C is likely to have a prooxidant effect which causes activation and even death of cells.

Therefore, the current study has developed an important assay that demonstrates the response of CD4+ T-cells to LPS-induced stimulation and further showed the effects of antioxidants in this process. The study was able to demonstrate that at this stage of HIV infection, CD4+ T-cells were able to respond to LPS-induced stimulation and antioxidants; therefore, they do not appear "exhausted" at this stage of the disease. However, it should be noted that LPS induced more death in the form of annexin V+/7-AAD+ staining in the HIV group than the control, suggesting that the cells may have been "primed" for death previously *in vivo*. Vitamin C alone did not inhibit LPS-induced activation in the HIV group as it did in the controls; suggesting that the use of vitamin C alone in HIV infection would not be of value. The combination of NAC with vitamin C produced the greatest level of inhibition of early apoptosis, suggesting a potential beneficial effect of this cocktail in the management of this stage of the infection. It is at this early stage of HIV infection that the "cocktail" is being most effective and this study has demonstrated beneficial effects of the cocktail in limiting immune activation and early apoptosis. This way immune cells can be rescued before irreversible damage to the cells occurs.

Limitations of the study were that specific tests for diagnosing underlying subclinical infections could not be performed and smoking and alcohol habits were not documented. Longitudinal cohort studies will be important to determine the value of intervention with the combination of anti-oxidants as described in this study, in HIV positive persons with CD4 counts >350 cells/mm^3.

5. Conclusion

This is an important assay that demonstrated the response of CD4+ T-cells to LPS-induced stimulation and showed the inhibitory effects of antioxidants. Certain combinations of antioxidants could be important in reducing the harmful effects of chronic immune activation and thereby limit CD4+ T-cell depletion. Importantly, the study showed that CD4+ T-cells of the HIV positive group responded better to a combination of vitamin C and NAC. Therefore, appropriate intervention at this asymptomatic stage could rescue the cells before exhaustion and senescence set in.

Conflict of Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

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Research Article

Prolactin Levels Correlate with Abnormal B Cell Maturation in MRL and MRL/lpr Mouse Models of Systemic Lupus Erythematosus-Like Disease

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Prolactin (PRL) plays an important role in modulating the immune response. In B cells, PRL enhances antibody production, including antibodies with self-specificity. In this study, our aims were to determine the level of PRL receptor expression during bone-marrow B-cell development and to assess whether the presence of high PRL serum concentrations influences absolute numbers of developing populations and disease outcome in lupus-prone murine models. We observed that the PRL-receptor is expressed in early bone-marrow B-cell; the expression in lupus-prone mice, which had the highest level of expression in pro-B cells and immature cells, differed from that in wild-type mice. These expression levels did not significantly change in response to hyperprolactinemia; however, populations of pro-B and immature cells from lupus-prone strains showed a decrease in the absolute numbers of cells with high PRL-receptor expression in response to PRL. Because immature self-reactive B cells are constantly being eliminated, we assessed the expression of survival factor BIRC5, which is more highly expressed in both pro-B and immature B-cells in response to PRL and correlates with the onset of disease. These results identify an important role of PRL in the early stages of the B-cell maturation process: PRL may promote the survival of self-reactive clones.

1. Introduction

Prolactin (PRL) is predominantly produced by the lactotropic cells of the anterior pituitary gland. However, it is also generated in extrapituitary sites, such as immune, decidual, mammary, epithelial, and fat cells [1–3]. PRL has multiple regulatory roles in reproduction, development, growth, osmosis, metabolism of carbohydrates and lipids, and the immune response. The PRL receptor is a member of the cytokine receptor superfamily [3–5]. Different isoforms of the PRL receptor have been found to be generated by alternative splicing at the 3′ end and variation in the intracellular domain length [3, 5, 6]. The PRL receptor is expressed in many immune cell types, mainly B cells, and also T cells, monocytes, macrophages, natural killer (NK) cells, and thymic epithelial cells [7, 8], and its activation induces transcriptional programs involved in various cellular functions such as proliferation, differentiation, and cytokine production. Hence, PRL has been implicated as a modulator of both cellular and humoural immunity [8–11].

Elevated serum PRL levels have been reported in several autoimmune diseases, including systemic lupus erythematosus (SLE) [12–14]. SLE is an autoimmune rheumatic disease. Serum samples from SLE patients characteristically have very strong reactivity to a variety of nuclear components, including DNA, RNA, histones, RNP, Ro and La. These antibodies form immune complexes that are deposited in the kidneys and may result in proteinuria and kidney failure. The presence of these autoantibodies indicates abnormalities in the activation and development of B cells [15, 16] and both B and T cells express the PRL receptor and secrete PRL [4, 17, 18]. SLE affects women of reproductive age at a 9 : 1 ratio compared to men and this gender bias has been attributed to the immunostimulatory properties of hormones. SLE symptoms typically begin or become exacerbated during pregnancy, when PRL serum levels are high. Nonphysiologically high serum concentrations of PRL also correlate with SLE symptoms [12, 14]. These findings have been reproduced in murine models of lupus (e.g., (NZB × NZW)F1 and MRL/lpr), in which the induction of hyperprolactinemia correlated with exacerbated disease symptoms, such as the early detection of autoantibodies, proteinuria and accelerated death [19, 20]. MRL-MpJFas^{lpr} (MRL/lpr) mice have a mutation in the Fas gene and develop a disease similar to SLE that is characterised by glomerulonephritis, vasculitis, splenomegaly, hypergammaglobulinemia, and the production of anti-dsDNA antibodies [21]. In this mouse strain, B cell elimination using an anti-CD79 antibody decreased the manifestation of SLE-like symptoms, demonstrating the importance of B cells in SLE physiopathology [22, 23].

B cells develop from hematopoietic stem cells in the bone marrow through a series of differentiation stages. The most immature cell committed to the B cell lineage is the B cell progenitor, also called the pro-B cell, which undergoes immunoglobulin heavy chain gene rearrangement and differentiates into a pre-B cell. Pre-B cells undergo immunoglobulin light chain gene rearrangement and develop into immature B cells. This latter population is tested for self-specificity first in bone marrow then in circulation and the spleen, where it is identified as transitional type I (T-1) B cells. These cells further develop into transitional type II (T-2) and type III (T-3) B cells to finally become mature B cells (follicular, and marginal zone cells) [24–27]. B cell antigen receptor assembly and testing for autoreactivity are the primary objectives of B cell development; therefore, the alteration of this maturation pathway results in a generation of B cell clones with the potential to cause autoimmune diseases.

Our group previously demonstrated that T-1 B cells express the highest level of PRL receptor of any other splenic B cell population. We also observed a significant increase in the absolute number of this B cell subset in mice that developed lupus during hyperprolactinemia [20]. Because T-1s represent the first subset of splenic B cells produced by bone marrow cells, it is possible that PRL targets earlier bone marrow developmental stages. Therefore, the aim of this study was to determine whether developing bone marrow B cells express the PRL receptor and whether development is altered in response to PRL sera levels that correlate with the onset of lupus in a murine model of this disease. Our results showed that all early bone marrow B cell populations express the PRL receptor. However, the expression was higher in pro-B and immature cells in lupus-prone mice, a pattern that differs from that of wild type mice. Increased levels of PRL hastened disease manifestations, which correlated with a reduction in the absolute number of maturing B cells. These results support an important role of PRL in the early stages of the B cell maturation process, thus helping to clarify its relevance to the development of SLE.

2. Materials and Methods

2.1. Mice. All studies were approved by the Animal Care Committee of Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubiran" and Hospital de Pediatría, Centro Médico Nacional Siglo XXI, IMSS (R-2011-785-015), and all of the mice experiments were performed in accordance with approved guidelines established by Mexico (Norma Oficial Mexicana NOM-062-ZOO-1999). The C57BL/6 mice were purchased from Harlan (Indianapolis, USA); the MRL/MpJ (MRL) and MRL/MpJFas^{lpr} (MRL/lpr) mice were purchased from The Jackson Laboratory (Maine, USA). Mice were housed in a pathogen-free barrier facility and were provided with sterile food and water *ad libitum*.

2.2. Antibodies. The following antibodies were used: APC-conjugated rat anti-mouse CD21 (7G6) from BD Biosciences (Mountain View CA, USA); FITC-conjugated rat anti-CD43 (eBioR2160), PE-conjugated rat anti-B220 (RA3-6B2), APC-conjugated rat anti-IgM (11/41), PE/Cy7-conjugated rat anti-CD23 (B3B4), PE-conjugated rat anti-CD93 (AA4.1), and FITC-conjugated rat anti-CD19 (eBioD3) from eBioscience (San Diego, CA, USA); goat anti-mouse PRL-R (E20) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and biotinylated swine anti-goat from Invitrogen (Carlsbad CA, USA). The biotinylated secondary antibody was detected using phycoerythrin-Cy5.5 conjugated streptavidin from BD Biosciences (Mountain View, CA, USA). AffiniPure F(ab)$_2$ fragment goat anti-mouse IgM was from Jackson ImmunoResearch (Baltimore, USA).

2.3. Purification of B Cells. Bone marrow (BM) cells were collected by flushing femoral shafts with cold RPMI (HyClone, Logan, Utah, USA) supplemented with 2% bovine serum albumin (BSA, US Biological, Swampscott, Ma, USA) and EDTA 2 mM (IBI Scientific, USA). After red blood cell depletion using lysis buffer (Sigma Aldrich, St. Louis, Missouri, USA), the cells were incubated with anti-B220 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and B cells were isolated by positive selection using a magnetic activated cell-sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany). After purification, >98% of the remaining cells were CD19$^+$ by flow cytometry.

2.4. Cell Sorting. B cells suspensions from BM were incubated with fluorescently labelled antibodies specific for CD43, B220, IgM, and CD23 in staining buffer (PBS with 0.5% BSA)

for 20 minutes at 4°C. The cells were washed, and the B cell ($B220^+$) subsets were separated according to the expression of the following surface markers: pro-B ($CD43^+$, $CD23^-$, and IgM^-), pre-B ($CD43^-$, $CD23^-$, and IgM^-), and immature cells ($CD43^-$, $CD23^-$, IgM^+). Cell sorting was performed using a FACSAria sorter with FACSDiva software (BD Bioscience, Mountain View, CA, USA). The purity of the sorted cells ranged from 95% to 98%.

2.5. Real Time RT-PCR. Total RNA was extracted from B cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and the RNA concentration was determined using UV spectrophotometry. SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was used to generate cDNA from 1 μg of total RNA according to the manufacturer's specifications. Genes of interest were amplified and quantified by real time RT-PCR using the LightCyclerTaqMan Master kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's specifications. Hydrolysis probes and primers were designed by Roche Diagnostic. The following primers were used: PRL receptor 5′-CAGTAAATGCCACGAACGAA-3′ (left), PRL receptor 5′-GAGGAGGCTCTGGTTCAACA-3′ (right), β-actin 5′-AAGGCCAACCGTGAAAAGAT-3′ (left), β-actin 5′-GTGGTACGACCAGAGGCATAC-3′ (right), BIRC5 (survivin) 5′-CCCGATGACAACCCGATA-3′ (left) and BIRC5 5′-CATCTGCTTCTTGACAGTGAGG-3′ (right). The final reaction volume was 10 μL. A LightCycler Instrument (Roche Diagnostic, Mannheim, Germany) was used to perform the RT-PCR reaction. The following RT-PCR conditions were used: 10 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 30 seconds at 60°C and 1 second at 72°C and 1 cycle of 30 seconds at 40°C. The β-actin gene was used as a normalisation control across samples. The relative expression of the PRL receptor and BIRC5 were calculated using the $2^{-\Delta CT}$ formula.

2.6. Induction of Hyperprolactinemia. Nine-week-old C57BL/6, MRL, and MRL/lpr mice (8 females per group) were subcutaneously injected with 200 μg of metoclopramide (Sigma Aldrich, St. Louis, MO, USA) in 100 μL of PBS for six weeks. A matched control group (C57BL/6, MRL and MRL/lpr) received PBS only (100 μL) over the same period. Urinary protein levels were assessed semiquantitatively using reagent strips for urinalysis (UriCheck-10, Axilab, Monterrey, NL, Mex). Serum samples obtained at the beginning and at the end of the experiments were kept at −35°C until they were assayed for PRL and anti-dsDNA antibodies.

2.7. Prolactin Assessment. Serum levels of PRL were detected by ELISA by coating 96-well MaxiSorp plates (Nunc, Rochester, NY, USA) overnight with 100 μL of 2 μg/mL anti-mouse PRL monoclonal antibody (clone 207518, R&D Systems, Minneapolis, MN, USA) in PBS at 4°C, block with 2% BSA, and incubat with the serum sample (1 : 10) overnight at 4°C. Recombinant mouse PRL (National Hormone and Peptide Program, NIH, donated by AF Parlow) was used as a standard. The plates were then incubated with 0.2 μg/mL biotinylated anti-PRL antibody (R&D Systems, Minneapolis MN, USA), avidin-conjugated alkaline phosphatase (Invitrogen, Carlsbad, CA, USA) and the enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich, St. Louis MO, USA) according to the manufacturer's instructions. The OD was measured at 405 nm using a Dynatech MR5000 ELISA reader.

2.8. Measurement of Anti-dsDNA Antibodies. Anti-dsDNA antibody serum concentrations were detected using ELISA. A 96-well MaxiSorp plate was coated with 100 μL of 5 μg/mL calf thymus dsDNA (Sigma Aldrich, St. Louis MO, USA) in bicarbonate buffer overnight at 4°C and was blocked with 2% BSA. The plates were then incubated for 1 h at 37°C with serum (1 : 50) or the anti-dsDNA antibody standard (clone 16-13, Chemicon International, Billerica MA, USA), followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) and substrate (5-bromo-4-chloro-3-indolyl phosphate). The OD was monitored at 405 nm using a Dynatech MR5000 ELISA reader.

2.9. Cell Surface Staining and Flow Cytometry. BM cells were incubated with fluorescently labelled antibodies for 20 minutes at 4°C in staining buffer (PBS with 0.5% BSA and 0.01% sodium azide). The cells were then washed and fixed in 2% paraformaldehyde (Sigma Aldrich, St Louis MO, USA). The data were acquired using a FACSAria flow cytometer and analysed with FlowJo software (Tree Star, Ashland, OR, USA).

2.10. Statistical Analysis. The data were analysed using standard statistical tests (mean value, SD, Student's t-test, and ANOVA) and the results are expressed as the mean ± SD. The level of significance was set at $P \leq 0.05$. All calculations were performed using SPSS 19 software.

3. Results

3.1. Expression of the PRL Receptor in B Cells. The pro-B, pre-B, and immature B cells from the bone marrow of C57BL6 wild-type mice were purified by flow cytometry to >95% purity (Figure 1(a)) and were assayed for the expression of PRL receptor mRNA and protein. Our results showed that all B cell developmental stages in the bone marrow express the PRL receptor. Immature B cells had the lowest relative mRNA expression (0.47 ± 0.04), which was significantly different ($P < 0.05$) compared to pre-B cells (1.04 ± 0.18) and pro-B cells (1.28 ± 0.10); there was no significant difference between pro-B and pre-B cells (Figure 1(b)). A similar expression pattern was observed at the protein level; immature B cells had the lowest PRL receptor expression (35.77 ± 9.41 MFI, mean fluorescence intensity), followed by pre-B cells (46.67 ± 6.05 MFI) and pro-B cells (119.30 ± 42.51 MFI) (Figure 2(a)). Thus, PRL receptor expression of pro-B cells is 2.6 times higher than that of pre-B cells and 3.3 times higher than that of immature cells. Figure 2(b) shows the flow cytometry histograms.

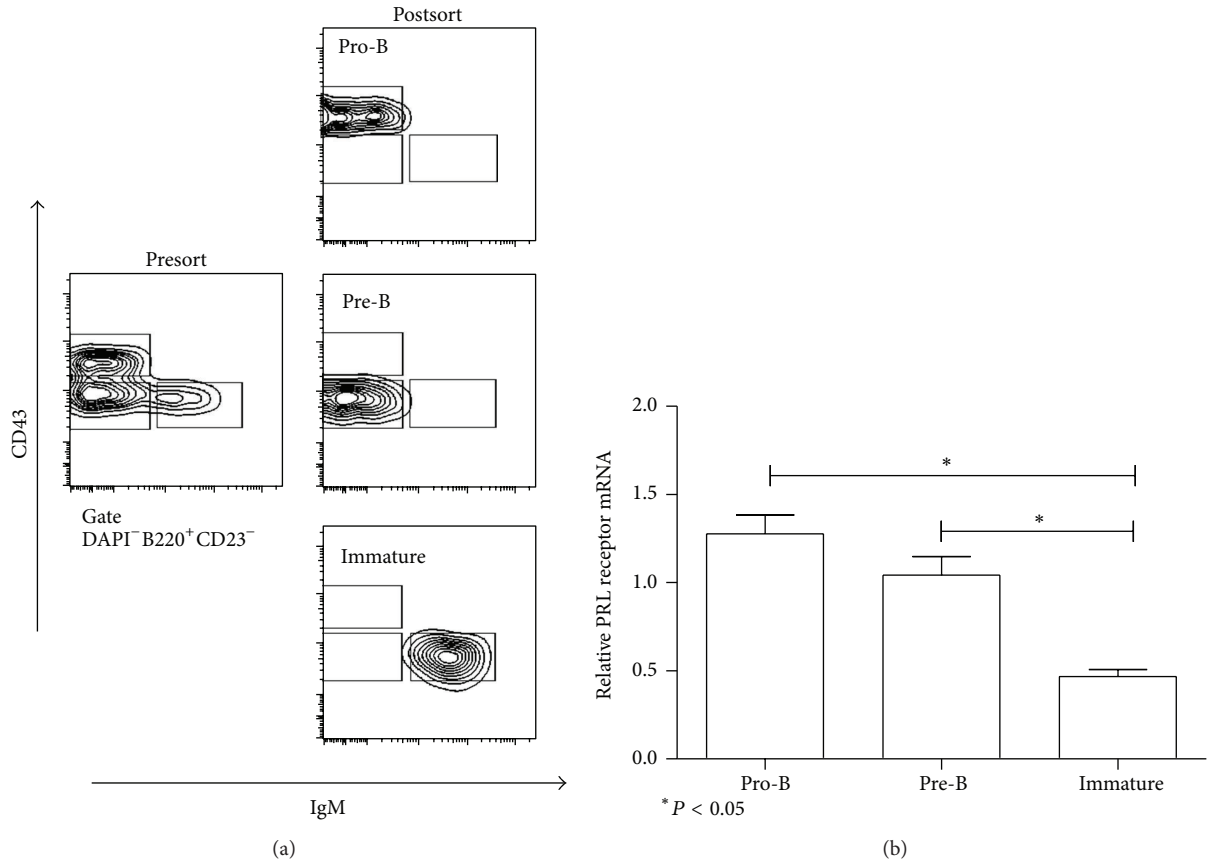

Figure 1: Purification of B cell by flow cytometry. B cells were purified from the BM of 9-week-old mice. (a) The cells were incubated with antibodies specific for B cell subsets, and the subsets were purified using flow cytometry, as detailed in Methods. The purity of the collected populations varied between 95% and 98%. A representative example of the purified B cells from wild-type C57BL/6 mice is shown. (b) Using RT-PCR, the PRL receptor mRNA expression was measured in the different subsets of bone marrow B cells (pro-B, pre-B and immature). The asterisks denote statistical significance with the *P* value shown.

3.2. Receptor Expression in Lupus-Prone Mice. Analysis of BM B cells from both lupus-prone strains (MRL and MRL/lpr) at 9 weeks of age (without disease manifestations) revealed a different PRL receptor expression pattern; pro-B cells had the highest level of PRL receptor expression (4.5- to 5.5-fold more than pre-Bs and 2.4- to 3-fold more than immature B cells). Thus, in lupus-prone mice, pro-B cells were followed by immature and pre-B cells as shown in Figure 2(c) (MRL: pro-B = 693.60 ± 46.56 MFI, pre-B = 153.40 ± 37.67 MFI, and immature = 288.00 ± 58.85 MFI) and Figure 2(d) (MRL/lpr: pro-B = 385.33 ± 43.70 MFI, pre-B = 77.66 ± 35.74 MFI, and immature = 128.50 ± 28.73 MFI); the differences between all populations were statistically significant. In this analysis, MRL mice showed the highest receptor expression, followed by MRL/lpr and C57BL/6 in all BM B cell populations.

3.3. Exacerbation of SLE by Hyperprolactinemia. Nine-week-old MRL/lpr, MRL, and C57BL/6 mice were treated with metoclopramide for six weeks to induce high levels of PRL and accelerate SLE symptoms. The serum concentrations of PRL for pretreatment (9 weeks), PBS-treated, and metoclopramide-treated mice were 4.2 ± 1.38, 3.80 ± 1.18, and 10.70 ± 1.23 ng/mL, respectively, for the C57/BL6 strain, 12.58 ± 1.99, 11.20 ± 1.81, and 26.27 ± 2.69 ng/mL, respectively, for the MRL strain, and 12.73 ± 2.25, 20.07 ± 2.75 and 34.51 ± 4.34 ng/mL, respectively, for the MRL/lpr strain. All strains had significantly increased PRL levels in sera in response to metoclopramide (hyperprolactinemia), while only MRL/lpr mice showed a significant increase after PBS treatment, which was likely age-related (15 weeks at the end of treatment); however, the PRL increase in the PBS group was lower than in the group treated with metoclopramide (Table 1).

Proteinuria and serum anti-dsDNA antibodies, two disease manifestations that mirror lupus symptoms, were measured and the concentrations were compared between MRL and MRL/lpr mice before and after treatment with metoclopramide or PBS. All mice had a significant increase in proteinuria in response to metoclopramide, while only the PBS-treated group of MRL/lpr mice also showed a significant increase correlating with the observed increase of PRL. This increase in proteinuria was also less dramatic than the increase observed in metoclopramide-treated mice (Table 1). Serum concentrations of anti-dsDNA IgG antibody in hyperprolactinemic MRL mice increased 4-fold compared to PBS

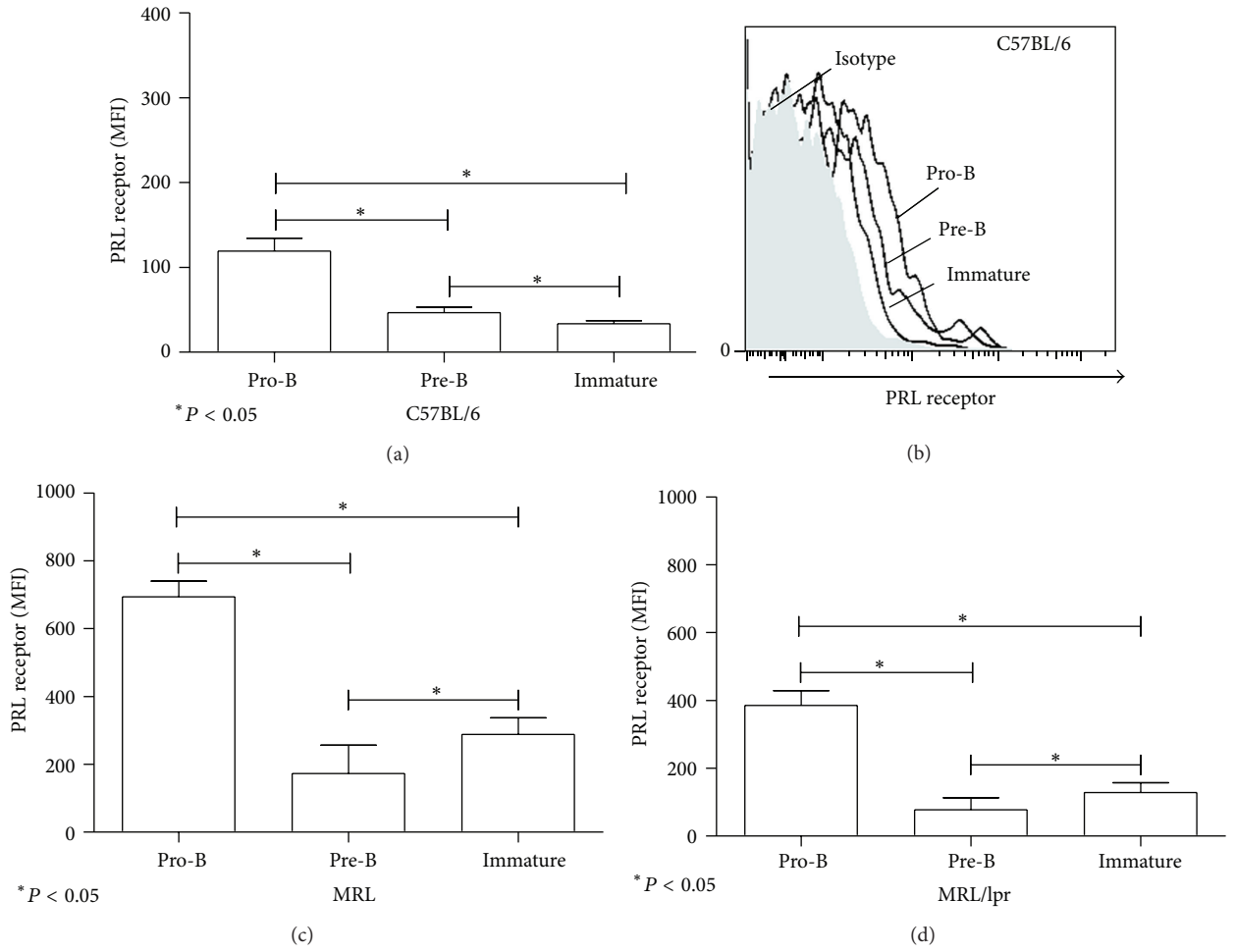

Figure 2: Expression of prolactin receptor in B cells. PRL receptor expression (MFI) was measured using flow cytometry from eight mice per strain. BM cells were labelled with anti-B220, anti-CD43, anti-CD23, anti-IgM, and goat anti-PRL receptor antibodies; the isotype control was labelled with anti-B220, anti-CD43, anti-CD23, anti-IgM, and goat unrelated antibodies. (a) Pro-B, pre-B and immature from C57BL/6 mice; (b) histograms of PRL receptor expression in B cells from BM; (c) pro-B, pre-B, and immature from MRL mice; (d) pro-B, pre-B, and immature from MRL/lpr mice. The asterisks denote statistical significance with the P value shown. The MFI values expressed in the graphs correspond to the MFI values minus the isotype control.

Table 1: SLE manifestations in mice with hyperprolactinemia.

Strain	C57BL/6			MRL			MRL/lpr		
Treatment	9 weeks	PBS	HyperPRL	9 weeks	PBS	HyperPRL	9 weeks	PBS	HyperPRL
PRL (ng/mL)	4.2 ± 1.3	3.8 ± 1.1	10.7 ± 1.2*	12.5 ± 1.9	11.2 ± 1.8	26.27 ± 2.9*	12.7 ± 2.2	20.0 ± 2.7*	34.5 ± 4.3*
Proteinuria (mg/mL)	0	0	0	10.1 ± 7.2	12.8 ± 5.6	121.6 ± 37.4*	13.5 ± 8.5	48.0 ± 18.6*	166.6 ± 23.5*
Ab anti-dsDNA (μg/mL)	0	0	0	0	5.9 ± 1.9*	22.9 ± 5.1*	2.5 ± 0.05	11.6 ± 1.2*	22.5 ± 5.1*

HyperPRL: hyperprolactinemia.
*ANOVA, $P < 0.05$.

treated mice (22.96 ± 5.11 and 5.94 ± 1.98 μg/mL, resp.). Similarly, MRL/lpr mice showed increased concentrations in hyperprolactinemic and PBS-treated mice (22.50 ± 5.10 and 11.60 ± 1.20 μg/mL). C57BL/6 mice not presented with proteinuria nor anti-dsDNA antibodies in any condition tested (Table 1). Taken together, these data show that increased PRL concentrations in serum correlates with the early onset of lupus symptoms in lupus-prone mouse strains.

3.4. Expression of the Prolactin Receptor in Mice with Hyperprolactinemia. We have previously reported that augmented PRL levels in serum result in higher levels of its receptor in B cell splenocytes [20]. When pro-B, pre-B, and immature cells were analysed, we found that hyperprolactinemia did not change PRL receptor expression in the wild-type control strain (Figure 3(a)). Similarly, there were no significant changes between the PBS and metoclopramide treated groups

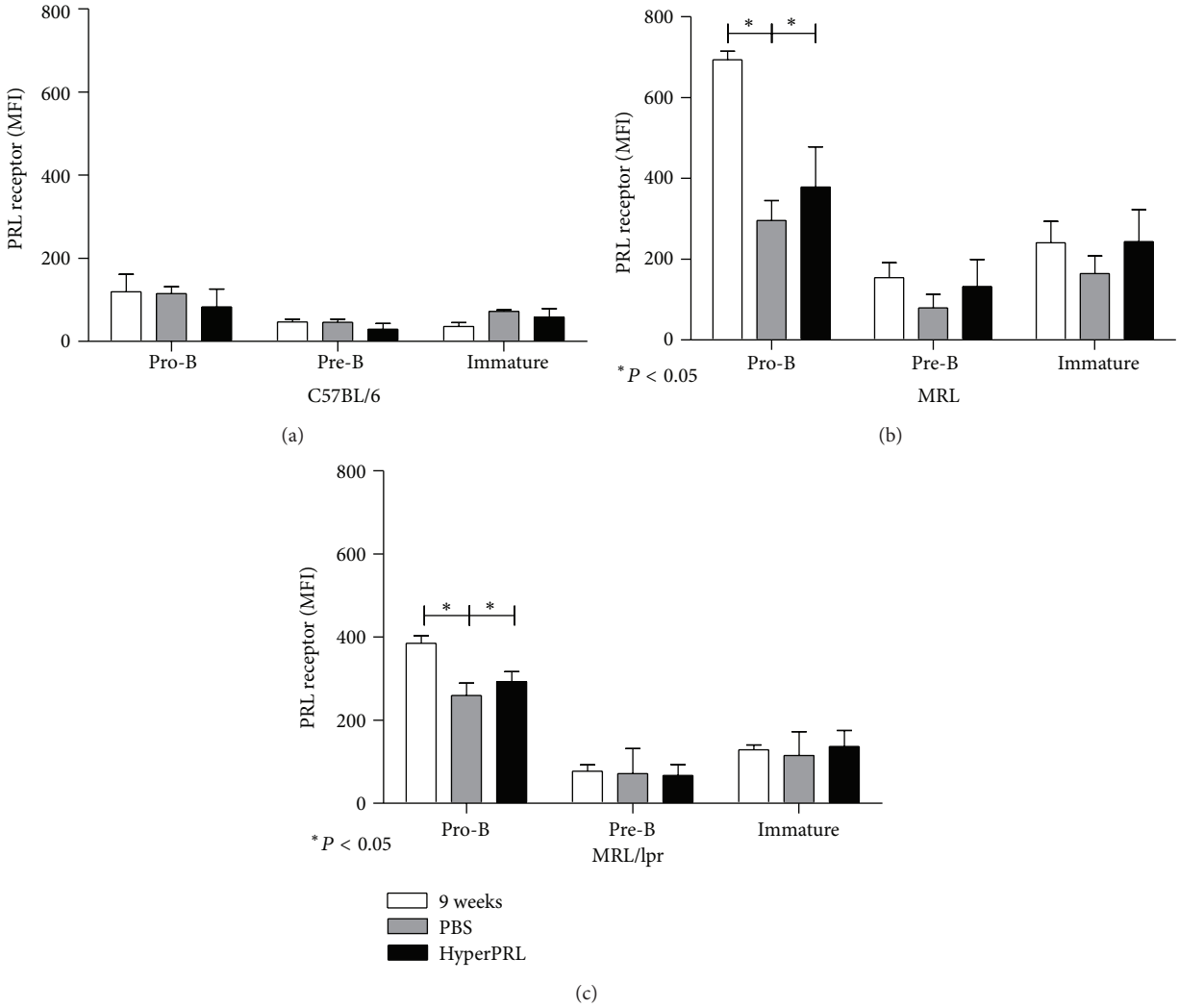

Figure 3: Prolactin receptor expression after the induction of hyperprolactinemia. The levels of PRL receptor protein (MFI) in the B cells from BM (pro-B, preB, and immature) were measured using flow cytometry. At the end of the treatment, the BM cells were labelled with anti-B220, anti-CD43, anti-CD23, anti-IgM, and goat anti-PRL receptor antibodies. (a) C57BL/6 mice; (b) MRL mice; (c) MRL/lpr mice. The asterisks denote statistical significance between populations with the P value shown. The MFI values expressed in the graphs correspond to the MFI values minus the isotype control.

in lupus-prone mice (MRL pro-B cells: 296.00 ± 49.46 and 378.66 ± 79.70 MFI, resp.; pre-B cells: 79.50 ± 33.04 and 132.50 ± 66.96 MFI, resp.; immature B cells: 164.60 ± 43.71 and 221.8 ± 84.03 MFI, resp.; MRL/lpr pro-B cells: 259.80 ± 29.78 and 292.83 ± 59.50 MFI, resp.; pre-B cells: 71.60 ± 27.00 and 67.00 ± 25.98 MFI, resp.; and immature B cells: 115.25 ± 28.63 and 136.60 ± 38.42 MFI, resp.) Figures 3(b) and 3(c). In contrast, an age-related significant decrease ($P < 0.05$) was observed in PBS-treated pro-B cells (MRL = 296.00 ± 49.46, MRL/lpr = 259.80 ± 29.78 MFI) compared with levels before treatment (MRL = 693.60 ± 46.56, MRL/lpr = 385.33 ± 43.78 MFI). This change was larger for MRL than for MRL/lpr mice. Thus, contrary to B cell splenocytes, there is no increase in PRL receptor levels in response to PRL in early B cell populations; instead, there is a decrease in this receptor with age. However, the levels of receptor expression of lupus-prone mice are still significantly higher than those of wild type control mice, especially for pro-B and immature B cells.

3.5. Estimation of Population Absolute Numbers. The absolute cell numbers of bone marrow B cell subsets were analysed as an indicator of possible effects of PRL in B cell development. In C57BL/6 control mice, we did not observe changes in any populations when treated with metoclopramide or PBS. Conversely, mice that developed lupus symptoms had a significant decrease in the absolute number of pro-B cells during hyperprolactinemia (MRL = $0.23 \pm 0.11 \times 10^6$ cells, MRL/lpr = $0.40 \pm 0.05 \times 10^6$) compared with PBS-treated mice (MRL = $0.33 \pm 0.12 \times 10^6$ cells, MRL/lpr = $0.53 \pm 0.04 \times 10^6$; Figure 4(a)). A decrease was also observed in immature B cells between metoclopramide-treated and PBS-treated mice (MRL = $0.15 \pm 0.07 \times 10^6$ and $0.30 \pm 0.08 \times 10^6$ cells, resp.;

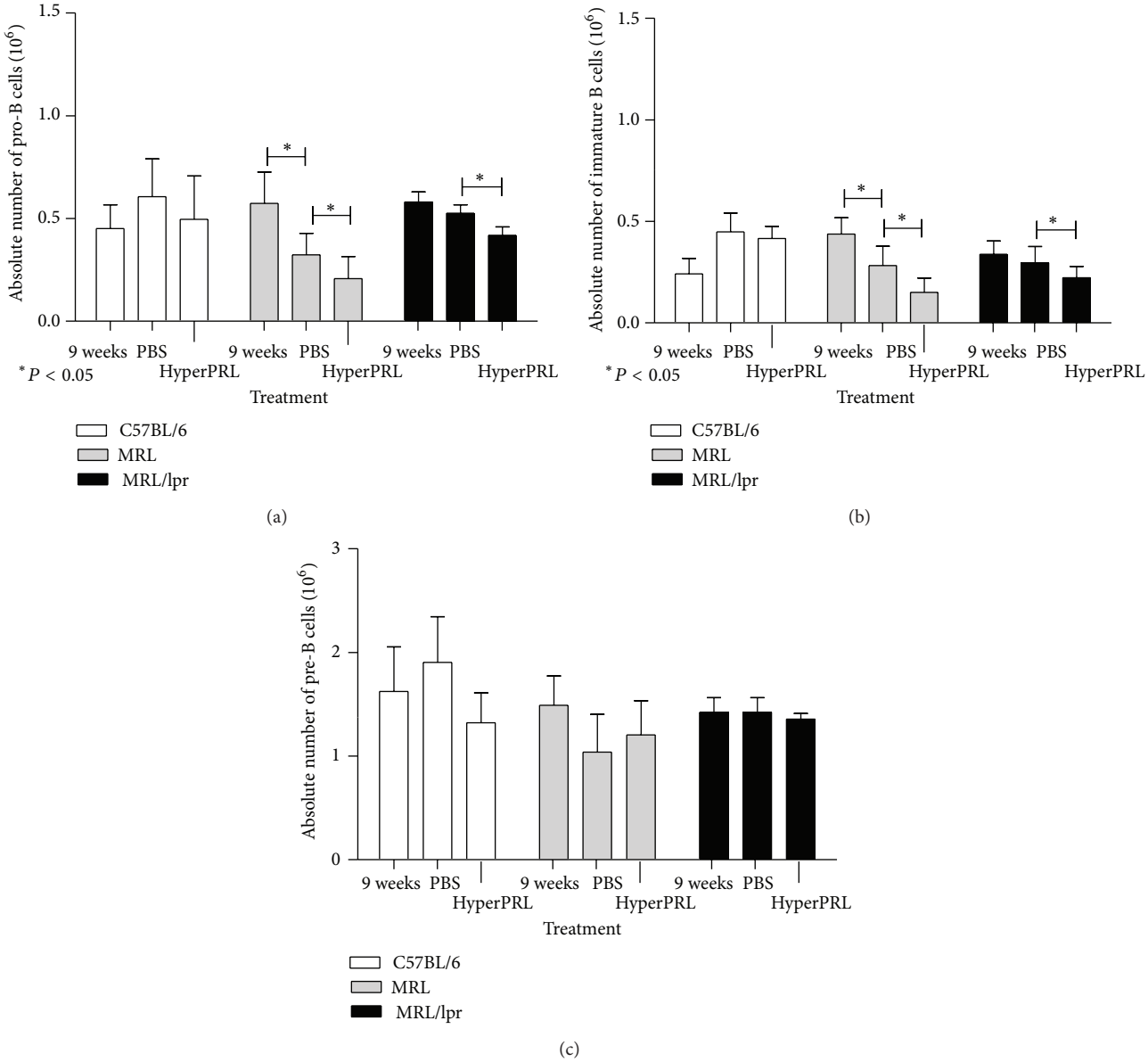

Figure 4: Absolute number of B cells from bone marrow after the induction of hyperprolactinaemia. Nine-week-old mice were treated with metoclopramide (200 μg/100 μL) to induce hyperprolactinaemia (HyperPRL) or PBS (100 μL) for 6 weeks, with eight mice per condition. At the end of the treatment, bone marrow cells were labelled with antibodies against B220, CD43, CD23, and IgM. (a) Graph of the absolute numbers of pro-B cells. (b) Graph of the absolute numbers of immature B cells. (c) Graph of the absolute numbers of pre-B cells. The asterisks denote statistical significance between populations with the P value shown.

MRL/lpr = $0.21 \pm 0.06 \times 10^6$ and $0.31 \pm 0.08 \times 10^6$ cells, resp.; Figure 4(b)). In contrast, the absolute number of pre-B cells was not affected by the hyperprolactinemic state, as shown in Figure 4(c). Therefore, a decrease in the absolute numbers of pro-B and immature B cells seems to correlate with these cells' basal PRL receptor expression (Figure 2). Although we did not observe further changes in receptor expression during hyperprolactinemia, the data in Figure 4 also support a PRL-mediated effect.

3.6. Increase in BIRC5 Expression in Immature Cells. Contrary to our previous observations in the spleen, numbers of BM B cells with higher levels of PRL receptor were reduced in response to PRL. Because self-reacting immature B cells are selected against, it is possible that PRL mediates the accelerated development of immature B cells. Using a gene expression microarray, we previously observed the upregulation of the BIRC5 gene, a survival factor, in total bone marrow cells from 9-week-old MRL/lpr mice cultured in the presence of PRL (manuscript in preparation). Therefore, we assessed whether the expression of this survival factor is altered during hyperprolactinemia. Bone marrow B cell populations were purified and assayed for BIRC5 expression by real time-RT-PCR. Figure 5 shows that BIRC5 expression does not vary in

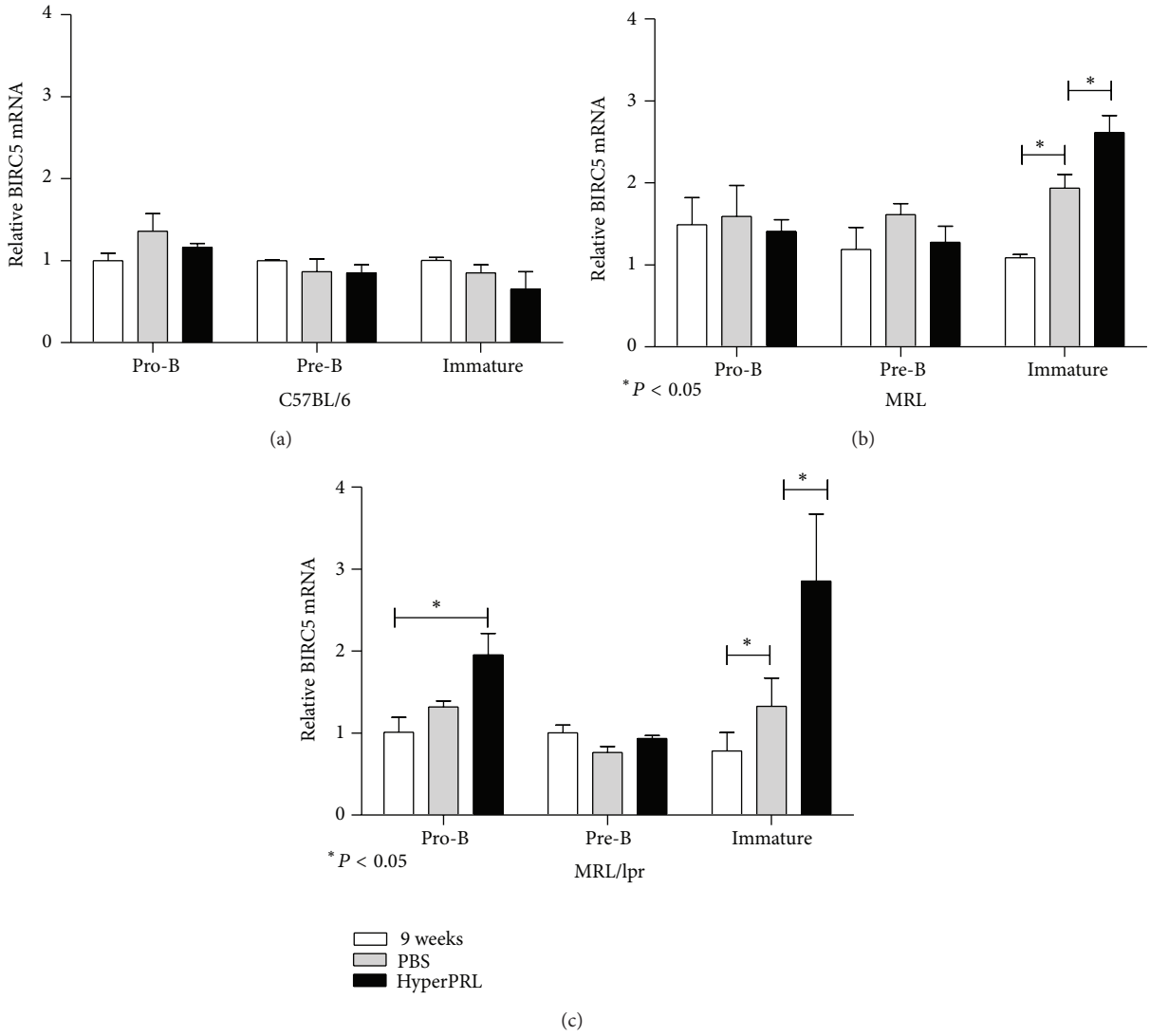

FIGURE 5: Increase in BIRC5 expression in immature cells. Nine-week-old mice were treated with metoclopramide (200 μg/100 μL) to induce hyperprolactinaemia (HyperPRL) or PBS (100 μL) for 6 weeks. At the end of the treatment, pro-B, pre-B and immature B cells were purified using flow cytometry in three independent experiments using three mice per experiment. Using RT-PCR, the BIRC5 mRNA expression was determined. (a) C57BL/6 mice; (b) MRL mice; (c) MRL/lpr mice. The asterisks denote statistical significance between populations with the P value shown.

the control mice (pretreatment = 1.00 ± 0.03; PBS-treated = 0.85 ± 0.10; and metoclopramide-treated = 0.65 ± 0.21; Figure 5(a)), while expression increased in both lupus-prone strains in response to PRL and aging primarily for immature cells (MRL: pretreatment = 1.08 ± 0.04; PBS-treated = 1.93 ± 0.16; metoclopramide-treated = 2.62 ± 0.20; MRL/lpr: pretreatment = 0.78 ± 0.22; PBS-treated = 1.32 ± 0.24; and metoclopramide-treated = 2.85 ± 0.81; Figures 5(b) and 5(c)) but also for pro-B cells in the MRL/lpr mice (pretreatment = 1.01 ± 0.18; PBS-treated = 1.32 ± 0.07; and metoclopramide-treated = 1.95 ± 0.26). Therefore, a correlation also exists between the expression of the survival factor BIRC5 and basal levels of the PRL receptor. Upregulation of antiapoptotic genes in immature B cells in response to PRL levels may be an important mechanism of escaping tolerance mechanisms and may explain the increase in autoantibodies in PRL-triggered SLE.

4. Discussion

Several studies have demonstrated the importance of PRL and B lymphocytes in the development of SLE [12–16]. We previously reported that all subsets of splenic B cells (T-1, T-2, T-3, follicular and marginal zone cells) express the PRL receptor, with the highest expression in the most immature subset (T-1s) in the lupus-prone mouse strains [20]. Because T-1s are directly produced from B cells developing in the bone marrow, we evaluated the expression of the PRL receptor in different bone marrow developmental stages (pro-B, pre-B and immature) as well as the response of these populations

to the pharmacological induction of a hyperprolactinemic state and correlated our observations with the course of SLE in MRL/lpr, MRL, and wild-type mice (C57BL/6). We found that bone marrow B cells also express the PRL receptor in all of the mouse strains analysed. However, in wild type mice, the expression of the receptor decreases as the B cell matures, while in strains that develop SLE, immature B cells together with pro-B cells have significantly higher levels of the PRL receptor compared to pre-B cells and their wild-type counterparts. The fact that the PRL receptor is expressed throughout all stages of the B cell developmental pathway supports the important role of PRL in B cell maturation and therefore in the function of B cell effectors.

It is known that increased PRL levels favour the appearance of SLE manifestations in NZB × NZW [28], *Sle3/5* R4A-γ2b C57BL/6 [29] and MRL/lpr mice [20]. In MRL and MRL/lpr strains, hyperprolactinemia correlates with premature SLE manifestations as well as increased receptor expression and aberrant B cell development in the spleen [20]. Although we did not observe differences in PRL receptor expression in response to PRL, we observed a reduced number of pro-B, and immature B cells with a high basal PRL receptor expression, indicating a PRL-mediated effect on B cell development. These data support an association between BM B cell maturation and disease progression. This observation is also supported by reports in which the SLE-prone strains MRL/lpr [30], BXSB [31], and NZB [32, 33] presented age-related anomalies in B cell development that were correlated with disease manifestations [34].

The main goal of B cell early development is to generate a functional BCR that is not self-reactive and B cell maturation is strongly dependent on either constitutively generated (tonic) signalling or ligand-induced BCR signalling [35]. Increased levels of IL-7 or the ectopic expression of antiapoptotic genes have resulted in the increased proliferation and survival of developing cells, but progression is arrested in the absence of these signals [36]. To our knowledge, there have been no previous *in vivo* studies of the effect of PRL on early B cell development. The reduced numbers of pro-B and immature B cells observed in response to PRL could be due to either accelerated developmental progression or increased negative selection.

Sexual hormones, such as oestrogens, regulate lymphopoiesis; pro-B lymphocytes are especially sensitive to high oestrogen concentrations, resulting in decreased numbers of these cells, where oestrogen can arrest lymphoid lineage differentiation [37, 38]. A similar PRL effect may explain the reduced numbers of pro-B cells observed during hyperprolactinemia. We also observed decreased numbers of immature B cells, which is an interesting observation because this is one of the main populations subjected to regulation against self-recognition. In transgenic mice in which BCR survival/tonic signals are favoured, for example, the SHIP knockout mice, accelerated development resulting in decreased immature/transitional populations has been observed [39]. PRL-triggered accelerated development throughout the immature stage would also explain our previous observation of the accumulation of T1 B cells in the spleen [20]. PRL may counteract mechanisms that prevent the self-reactivity of immature B cells, facilitating their rapid exit from bone marrow and the feeding of the splenic T-1 pool. Therefore, the results by Morales et al. [40] regarding PRL-induced B cell development argue for B cell maturation coordinated by the BCR together with environmental signals. These latter signals, such as PRL-induced signals, are also critical and may shape the B cell repertoire in response to different physiological stages.

Ligand-induced BCR signals are often associated with triggering the elimination of autoreactive clones at immature and transitional stages. PRL receptor signalling is known to increase the expression of antiapoptotic genes, such as Bcl-2 [41, 42], and T-1 B cells from hyperprolactinemic BALB/c mice are more resistant to apoptosis [43]. In line with these observations, we found that PRL increases the expression of the BIRC5 (survivin) gene (see Supplementary Figure 1 in supplementary material available online at http://dx.doi.org/10.1155/2013/287469), which belongs to a family of apoptosis inhibitors (IAP) [44, 45]. Survivin plays an important role in cell cycle entry/progression, maturation, and the inhibition of apoptosis as well as increasing the survival of hematopoietic stem/progenitor cells [46–50]. An increased expression of BIRC5 in immature B cells was found only in the SLE mice in response to hyperprolactinemia. Furthermore, BM B cells incubated with an anti-IgM antibody have increased survival rates in hyperprolactinemic conditions (Supplementary Figure 2). Taken together, these data indicate an important effect of PRL on B cell development, both favouring positive selection and counteracting mechanisms against self-specificity. In this scenario, increased PRL levels would result in the maturation of B cell clones with self-reactivity and an increased risk for developing autoimmune diseases. It will be interesting to determine the molecular mechanisms by which PRL and PRL receptors interfere with B cell maturation and tolerance, which will aid in the rational design of targeted therapy with potential applications for both autoimmunity and immunodeficiencies.

5. Conclusions

The PRL receptor is expressed by pro-, pre-, and immature B cells in the bone marrow suggesting an important role for PRL in early B cell development. In agreement, both populations with increased receptor expression, pro-Bs and immatures, upregulate the expression of survival factor BIRC5 in response to PRL. This might be an important mechanism for breakdown of tolerance, since PRL-enhanced BIRC5 expression correlated with an early onset of lupus symptoms.

Authors' Contribution

Legorreta-Haquet Maria Victoria and Flores-Fernández Rocio contributed equally to this work.

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Clinical Study

Effect of Selective Serotonin Reuptake Inhibitors and Immunomodulator on Cytokines Levels: An Alternative Therapy for Patients with Major Depressive Disorder

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Major depressive disorder (MDD) is a psychiatric illness that presents as a deficit of serotonergic neurotransmission in the central nervous system. MDD patients also experience alterations in cortisol and cytokines levels. Treatment with selective serotonin reuptake inhibitors (SSRIs) is the first-line antidepressant regimen for MDD. The aim of this study was to determine the effect of a combination of SSRIs and an immunomodulator—human dialyzable leukocyte extract (hDLE)—on cortisol and cytokines levels. Patients received SSRIs or SSRIs plus hDLE. The proinflammatory cytokines IL-1β, IL-2, and IFN-γ; anti-inflammatory cytokines IL-13 and IL-10; and 24-h urine cortisol were measured at weeks (W) 0, 5, 20, 36, and 52 of treatment. The reduction in cortisol levels in the SSRI-treated group was 30% until W52, in contrast, the combined treatment induced a 54% decrease at W36. The decline in cortisol in patients who were treated with SSRI plus hDLE correlated with reduction of anti-inflammatory cytokines and increases levels of proinflammatory cytokines at the study conclusion. These results suggest that the immune-stimulating activity of hDLE, in combination with SSRIs, restored the pro- and anti-inflammatory cytokine balance and cortisol levels in depressed patients versus those who were given SSRIs alone.

1. Introduction

Clinical and epidemiological studies have established that major depressive disorder (MDD) is a cause of chronic stress [1, 2]. The World Health Organization asserts that MDD will be the second leading cause of incapacity worldwide by 2030 [3], representing a tremendous public health problem with high economic costs [4]. Increased stress levels affect the duration and extent of symptoms of depression [5]. One of the most common clinical features of MDD is the development of hypothalamic-pituitary-adrenal (HPA) axis abnormalities [6, 7]. Chronic hyperactivity of the HPA axis induces hypercortisolism, which affects the nervous, endocrine, and immune systems [8]. The HPA axis function is upregulated by proinflammatory cytokines through the brain receptors for this soluble molecules, expressed mostly at hypothalamus [9]. This stimulation induces a rise on circulatory levels of glucocorticoids that decreases the inflammatory systemic

Table 1: Demographic characteristics in depressed subjects and healthy volunteers.

	Age (years)	Gender (male/female)	BMI (kg/m^2)	Education (years)	Family history (positive/negative)	First episode	Recurrent episode
Healthy volunteers ($n = 30$)	32 ± 6	10/20	24.3 ± 0.4	15 ± 3	NA	NA	NA
Patients/SSRIs ($n = 31$)	35 ± 9	10/21	24.6 ± 0.7	13 ± 2	10/21	15	16
Patients/SSRIs plus DLE ($n = 34$)	33 ± 9	6/28	24.0 ± 4.0	12 ± 3	16/18	22	12

Values are given as mean ± standard deviation. Education refers to the number of years of schooling. Family history is expressed as the number of patients with depressive antecedents (positive) versus the number of patients without depressive antecedents (negative). NA: nonapplicable. BMI: body mass index.

effects induced by cytokines and diminishes the release of CRH at hypothalamus, generating a negative feedback loop.

Various studies have linked variations in cytokine and cortisol levels in MDD [10]; one of the first studies of the neuromodulatory effects of cytokines reported the induction of depressive symptoms by therapeutic IL-2 and IFNs [11]. Proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, also elicit adverse behavioral effects (fatigue, soporific effects) and symptoms of anxiety and depression [12].

MDD patients have high levels of cortisol in bodily fluids, such as saliva, blood, cerebrospinal fluid, and urine [13–15]. In addition, patients with MDD also experience deficits in serotonergic neurotransmission in the central nervous system [16], which is why they are treated successfully with selective serotonin reuptake inhibitors (SSRIs).

SSRIs are designed to compensate for alterations in serotonin levels (5-HT) and are usually administered over 1 year [17]. The HPA axis is restored, following clinical responses to treatment with SSRIs—SSRIs decrease circulating cortisol levels by reestablishing the downregulated glucocorticoid receptor, increase serotonin levels in circulation by blocking its reuptake receptor (5-HTT), and modify circulating cytokine profiles by receptors for neurotransmitters, glucocorticoids, and cytokines [15, 18, 19].

Our group has reported that without pharmacological intervention, MDD patients have a predominantly anti-inflammatory cytokine profile that is associated with high cortisol levels [20, 21] and that the administration of SSRIs for 52 weeks reverses the symptoms of MDD and modifies the cortisol and cytokines altered levels without restoring to normal levels [20].

Controlled trials have reported that 30% to 40% of MDD patients become resistant to pharmacological treatments due to medical comorbidities, unavailability of appropriate services, and poor adherence to therapies [22]. Despite advances in SSRIs, the management of MDD still requires pharmacological modalities that restore the immunological and endocrine imbalance in depressed patients.

Although the mechanisms of action are not fully understood, we used a dual pharmacological therapy SSRI plus human dialyzable leukocyte extracts (hDLEs) and measured cortisol and cytokines levels for 52 weeks of followup in depressed patients. hDLEs comprise many peptide sequences below 17 kDa [23], and hDLEs have been used widely as an adjuvant for patients with infectious diseases and deficient cell-mediated immune responses [24, 25]. DLEs stimulate the production of proinflammatory cytokines, including TNF-α and IL-6 [26], but their effects on the endocrine and immune dysfunction in MDD patients are unknown. This study examined the efficacy of hDLEs in reverting endocrine and immune alterations in adult outpatients with MDD.

2. Materials and Methods

2.1. Patients. The outpatient clinic of Instituto Nacional de Psiquiatria "Ramón de Fuente," Mexico City, assessed 434 individuals and recruited 65 Mexican patients. Patient recruitment was made according to the clinical experimental procedures set out in the INPRF-NC092318.0 research protocol, approved by the ethics committee of the Instituto Nacional de Psiquiatría, México. All subjects were diagnosed by psychiatrists who applied the Mini-International Neuropsychiatric Interview, a standardized diagnostic interview that is based on DSM-IV-TR criteria. Clinical status was measured using the Hamilton Depression Scale (HDRS) and Beck Depression Inventory (BDI). Patients who met the inclusion criteria were free of antidepressants for at least 3 weeks before the study. Each subject underwent laboratory screens to rule out other medical illnesses. After receiving a detailed explanation of the study aims, all participants signed written consent forms.

All patients were administered SSRIs (19 fluoxetine, 7 paroxetine, and 5 Sertraline) or SSRIs plus hDLEs (23 fluoxetine, 9 paroxetine, 1 sertraline, and 1 escitalopram). All patients were evaluated monthly by their psychiatrist, based on the HDRS and BDI. Blood and urine samples were obtained at weeks (W) 0, 5, 20, 36, and 52 of treatment. Figure 1 shows the total number of patients who were evaluated throughout the study, the changes in pharmacological treatment, and the causes for protocol withdrawal. The patients' demographics are shown in Table 1.

2.2. Drugs. The doses of SSRIs (mg/day) were as follows: fluoxetine, 20; paroxetine, 20; sertraline, 100; and escitalopram, 10. SSRI doses were established for each patient by his physician and adjusted when it was necessary throughout the study; the drugs were paid for by the patients (Figure 1).

hDLEs (Tranferon) were kindly provided by the Proyecto Factor de Transferencia, Escuela Nacional de Ciencias Biológicas, Instituto Politecnico Nacional (Mexico City, MX). For the group that was given SSRIs plus hDLEs, 30 units of hDLEs were administered to each patient for the 52 weeks of followup as follows: 3 units in week 1, 2 units in week 2, 1 unit each in weeks 3 and 4, and 1 unit every 2 weeks from Week 5 to the end of the study, as described [27].

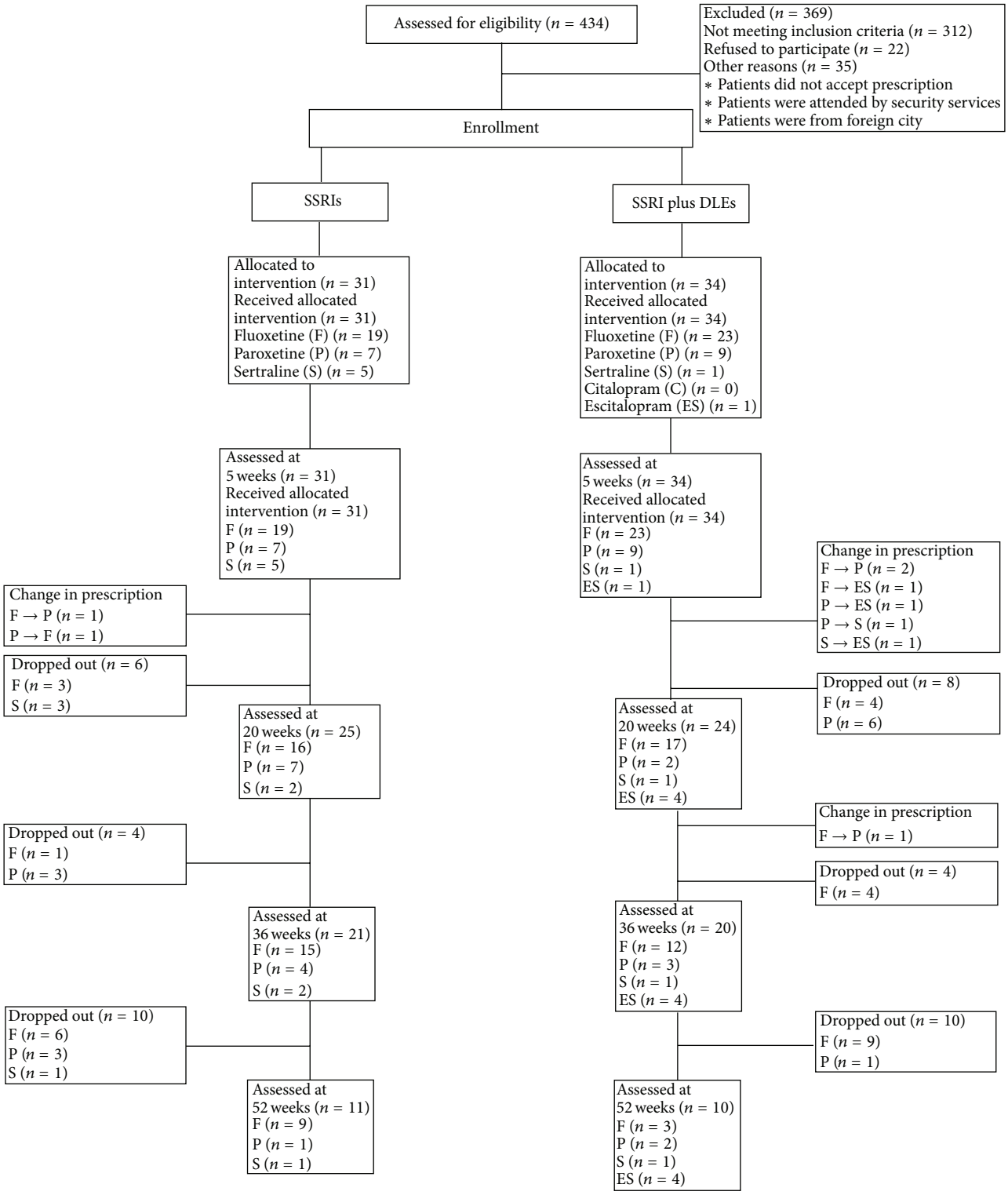

Figure 1: Flowchart of 52 week SSRIs and SSRIs plus hDLEs treatment in MDD patients. The numbers in parenthesis refer to the number of patients evaluated throughout the study, the changes in patient numbers for a pharmacological treatment, and the changes in patient numbers for treatment types withdrawn from the protocol. Change in prescription refers to the symbol >.

2.3. Sample Collection and Measurement of Cortisol and Cytokines by RIA and ELISA. Participants were instructed to collect their urine for 24 hours, in which total cortisol was measured by radioimmunoassay (RIA). Circulatory levels of IL-1β, IL-2, IFN-γ, IL-4, IL-10, and IL-13 were measured in serum from 30 mL of peripheral blood. Blood and 24 h urine samples were collected at W0, W5, W20, W36, and W52.

2.4. ELISA of Cytokines. Human IL-1β, IL-2, IFN-γ, IL-4, IL-10, and IL-13 were measured by ELISA. The ranges of detection were (pg/mL): IFN-γ, 5–1000; IL-1β, 3.91–250; IL-2, 78–1250; IL-4, 31.25–2000; IL-10, 4–2000; and IL-13, 312–5000. Primary antibodies were diluted to 4 μg/mL (except for anti-IL-10 and anti-IFN-γ; 8 μg/mL), 100 μL of which was added to each well of a 96-well plate.

Nonspecific binding was prevented with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Standards and samples were pipetted, and secondary antibodies were added to the plate. The secondary antibody concentrations were as follows (ng/mL): IFN-γ, 125; IL-1β, 100; IL-2, 25; IL-4, 50; IL-10, 400; and IL-13, 400. The immunoreactions were visualized with streptavidin-peroxidase solution using tetramethylbenzidine as substrate. The colorimetric reaction was stopped with sulfuric acid, and optical density was measured on a spectrophotometer (λ = 492 nm). The intra- and interassay variability was less than 5% and 7%, respectively.

2.5. Statistical Analysis. Data were analyzed using Prisma 6 for Mac OS X (GraphPad Software, La Jolla, CA, USA, http://www.graphpad.com/). Differences between means were analyzed using the homogeneity of variance test, followed by one-way ANOVA with Bonferroni's post hoc correction. Significant differences were calculated by comparing patients before antidepressant treatment (W0) and the healthy volunteers (HVs). Then, the values before antidepressant treatment (W0) were compared with those during the treatment (W5, W20, W36, and W52) in MDD patients. Finally, the data between patients at W52 and the HV were compared. Statistical significance was set to $P < 0.05$.

3. Results

3.1. Clinical and Psychiatric Assessment. Clinical and laboratory parameters, as measured by the Institute's clinical laboratory, such as complete blood count, blood chemistry, thyroid function test (T3, T4, and TSH), and complete urinalysis, fell within normal ranges of reference values in MDD patients and healthy volunteers; no parameter differed significantly between groups (data not shown). Table 1 shows the demographics and data on recurrence for the study participants, and Table 2 shows the scores on the psychiatric scales. At W0, MDD patients had an HDRS score of (SSRIs = 20 ± 2 and SSRIs plus hDLEs = 24 ± 4 points). Clinical remission attained at W20, at which point the HDRS score was SSRIs (3.3 ± 2 points) and SSRIs plus hDLEs (2.8 ± 3 points), and was maintained until the end of the study.

3.2. Cortisol. The concentrations of urinary cortisol in healthy volunteers and depressed patients before and throughout the 52 weeks of treatment are shown in Figure 2. Cortisol levels showed significant changes ($F_{[1,10]} = 50.7$, $P < 0.0001$). In MDD patients before treatment (W0) the hormone levels were significantly higher (SSRIs = 18 ± 3 and SSRIs plus hDLEs = 18 ± 4) than in healthy volunteers (6 ± 2). By post hoc comparison showed differences during treatments (SSRIs = 12 ± 3 and SSRIs plus hDLEs = 21 ± 5) at W5, (SSRIs = 17 ± 4 and SSRIs plus hDLEs = 8.5 ± 1.8) at W20, (SSRIs = 18 ± 4 and SSRIs plus hDLEs = 9 ± 0.6) at W36, and (SSRIs = 12 ± 3 and SSRIs plus hDLEs = 7 ± 1.8) at W52, when compared with W0 higher (SSRIs = 18 ± 3 and SSRIs plus hDLEs = 18 ± 4). At the end of the study, the cortisol level in MDD patients with SSRIs plus hDLEs treatment was not significantly different from that in the HVs. In contrast,

Table 2: Hamilton depression rate score in depressive patients.

	W0	W5	W20	W36	W52
Patients/SSRIs ($n = 31$)	20 ± 2 ($n = 31$)	10 ± 2 ($n = 31$)	3.3 ± 2 ($n = 25$)	4 ± 2 ($n = 21$)	2.6 ± 1.9 ($n = 11$)
Patients/SSRIs plus hDLE ($n = 34$)	24 ± 4 ($n = 31$)	13 ± 4 ($n = 34$)	2.8 ± 3 ($n = 24$)	2 ± 1 ($n = 20$)	2.4 ± 1 ($n = 10$)

Values are given as mean ± standard deviation.

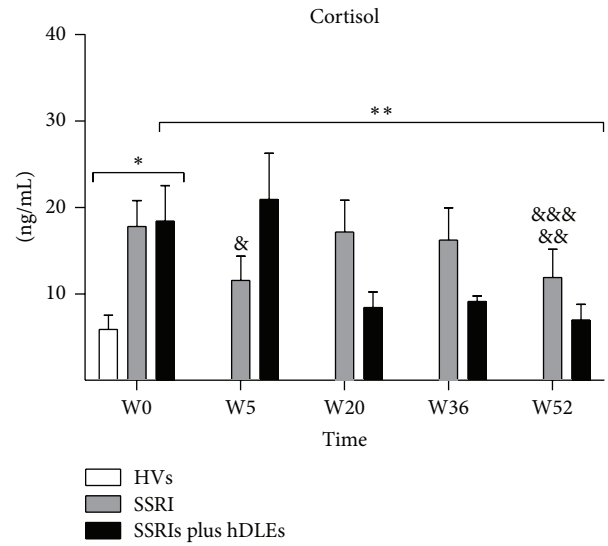

Figure 2: 24 h urinary cortisol levels detected by RIA in healthy volunteers and MDD patients. Patients were treated with SSRIs or SSRIs plus hDLEs during 52 weeks of study. The statistical analyses were as follows. First, the patient group before antidepressant treatment (W0) was compared with the control group (HVs). Second, the values before the antidepressant treatment (W0) were compared with those during the treatment (W5, W20, W36, or W52) in patients. Third, the data of patients at W52 versus HVs were compared. $^{*}P < 0.0001$, significant difference before treatment versus HVs. $^{**}P < 0.001$ between SSRIs plus hDLEs treatment at W5, W20, W36, and W52 versus W0. $^{\&}P < 0.0001$ between SSRIs treatment at W5 versus W0. $^{\&\&}P < 0.05$ between SSRIs treatment at W52 versus W0. $^{\&\&\&}P < 0.05$ between SSRIs treatment at W52 versus HVs. Data are expressed as mean ± SD. HVs: healthy volunteers; MDD: major depression disorder; W: weeks.

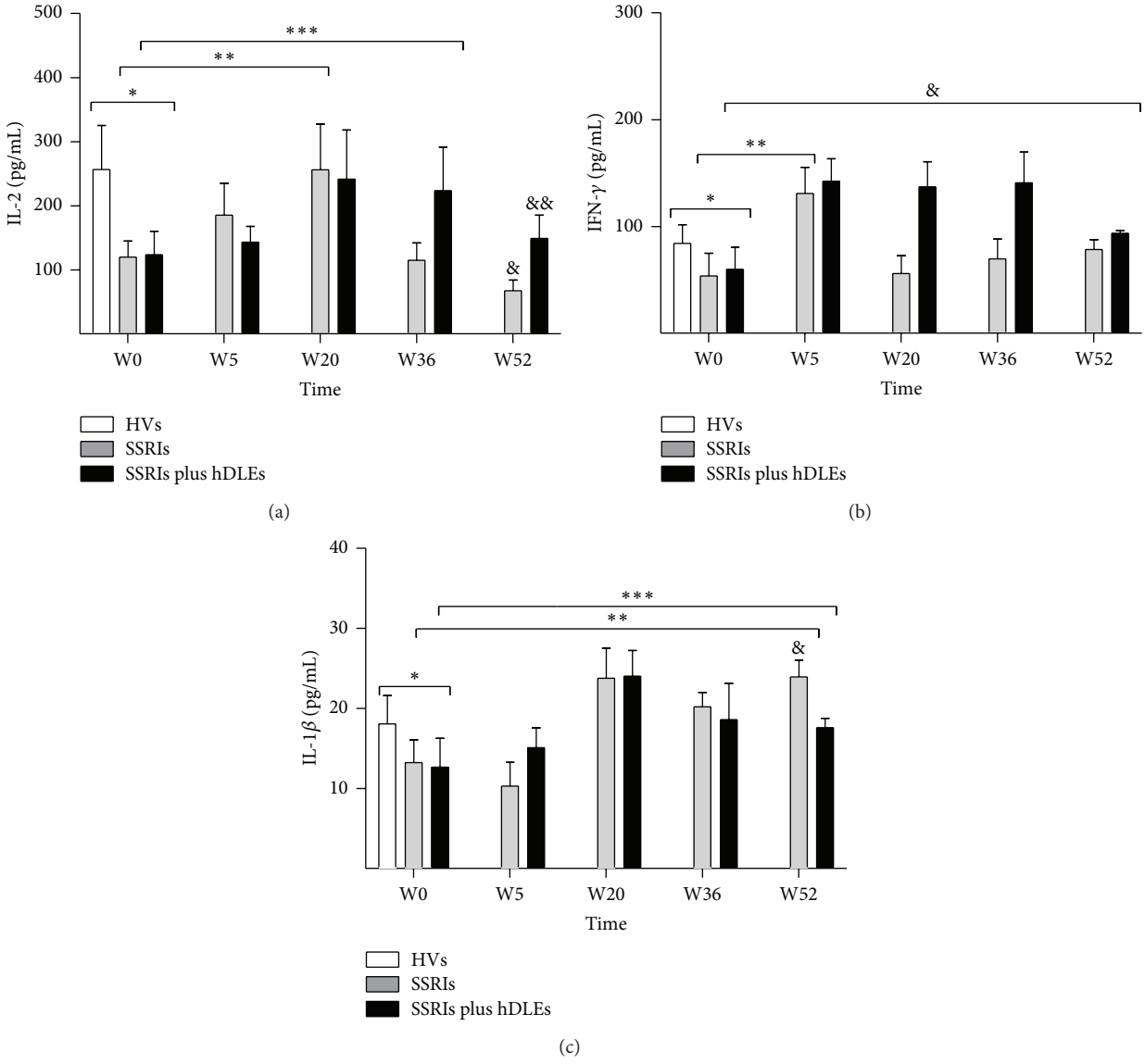

Figure 3: Serum proinflammatory cytokines detected by capture ELISSA assay in HVs and MDD patients. Patients were treated with SSRIs or SSRIs plus hDLEs during 52 weeks of study. The statistical analyses were as follows. First, the patient group before antidepressant treatment (W0) was compared with the control group (HVs). Second, the values before the antidepressant treatments (W0) were compared with those during the treatment (W5, W20, W36, or W52) in patients. Third, the data of patients at W52 versus HVs were compared. IL-2 (a), IFN-γ (b), and IL-1β (c): $^{*}P < 0.0001$, significant difference before treatments versus HVs. IL2: $^{**}P < 0.0001$ between SSRIs treatment at W5 and W20 versus W0. $^{***}P < 0.001$ between SSRIs plus hDLEs treatment at (W) 5, 20, and 36 versus W0. $^{\&}P < 0.0001$ between SSRIs treatment at W52 versus HVs. $^{\&\&}P < 0.001$ between SSRIs plus hDLEs treatment at W52 versus HVs. IFN-γ: $^{**}P < 0.0001$ between SSRIs treatment at W5 versus W0. $^{\&}P < 0.0001$ between SSRIs plus hDLEs treatment at (W) 5, 20, 36, and 52 versus W0. IL-1β: $^{**}P < 0.001$ between SSRIs treatment at (W) 5, 20, 36, and 52 versus W0. $^{***}P < 0.0001$ between SSRIs plus hDLEs treatment at (W) 5, 20, 36, and 52 versus W0. $^{\&}P < 0.0001$ SSRIs treatment at W52 versus HVs. Data are expressed as mean ± SD. HVs: healthy volunteers; MDD: major depression disorder; W: weeks.

the cortisol levels in MDD patients with SSRIs treatment showed a significant reduction when compared with healthy volunteers (Figure 2).

3.3. Proinflammatory Cytokines Profile. Variations in circulating proinflammatory cytokine levels were determined by ELISA using antibodies against cytokines specific. The levels expressed in pg/mL are shown in Figure 3(a) (IL-2), Figure 3(b) (IFN-γ), and Figure 3(c) (IL-1β).

3.3.1. IL-2. IL-2 showed significant differences between the HVs and MDD patients before and during the SSRIs and SSRIs plus hDLEs treatments ($F_{[1,10]} = 30.3$, $P < 0.0001$). Prior to treatment, MDD patients had decreased IL-2 levels when compared with the HVs (SSRIs = 120 ± 25, SSRIs plus hDLEs = 123 ± 37 versus HV = 256 ± 69). MDD patients showed increases at W5 (SSRIs = 185 ± 49 and SSRIs plus hDLEs = 142 ± 24), W20 (SSRIs = 256 ± 71 and SSRIs plus hDLEs = 241 ± 71), W36 (SSRIs plus hDLEs = 224 ± 68), and

W52 (SSRIs plus hDLEs = 149 ± 36). In contrast, IL-2 was significantly decrease with SSRIs (115 ± 27) at W36 and W52 (67 ± 16) (Figure 3(a)).

3.3.2. IFN-γ. IFN-γ levels fluctuated during the treatments and showed significant changes ($F_{[1,10]} = 66.9$, $P < 0.0001$). Before treatment, patients had lower IFN-γ levels (SSRIs = 54 ± 21 and SSRIs plus hDLEs = 60 ± 21) compared with healthy volunteers (84 ± 17). At W5 (SSRIs = 131 ± 24 and SSRIs plus hDLEs = 142±21), W20 (SSRIs = 66 ± 16 and SSRIs plus hDLEs = 137 ± 24), W36 (SSRIs = 69 ± 18 and SSRIs plus hDLEs = 141 ± 28), and W52 (SSRIs = 78 ± 9 and SSRIs plus hDLEs = 94 ± 3). At the end of treatment, IFN-γ levels were comparable with those of healthy volunteers (Figure 3(b)).

3.3.3. IL-1β. IL-1β showed contrast and significant changes ($F_{[1,10]} = 56.2$, $P < 0.0001$). Before treatment, circulating levels of IL-1β were significantly lower in patients (SSRIs = 13±3 and SSRIs plus hDLEs = 13±4) versus healthy volunteers (18±4). At W5, IL-1β increased with SSRIs plus hDLEs (15±2) and fell with SSRIs (10 ± 3) compared to W0. Both treatments showed increases at W20 (SSRIs = 24 ± 4 and SSRIs plus hDLEs = 24 ± 3), W36 (SSRIs = 19 ± 2 and SSRIs plus hDLEs = 22 ± 1), and W52 (SSRIs = 19 ± 4 and SSRIs plus hDLEs = 18±1). At the end of the followup, IL-1β levels of patients were comparable with those of healthy volunteers (Figure 3(c)).

3.4. Anti-Inflammatory Cytokines Profile. Variations in circulating anti-inflammatory cytokine levels are shown in Figure 4(a) (IL-4), 4B (IL-10), and 4C (IL-13).

3.4.1. IL-4. The values of circulating levels of IL-4 in healthy volunteers were below the range of sensitivity of the assay (31.25–1000 pg/mL). Thus, we considered these values nondetectable (ND). Before treatments, the patients showed significant increases in IL-4 (SSRIs = 45 ± 16 and SSRIs plus hDLEs = 42 ± 17). Changes in IL-4 levels fluctuated during treatments ($F_{[1,5]} = 18$, $P < 0.0001$). Patients treated with SSRIs had increased cytokine levels at followup study. In contrast, SSRIs plus hDLEs treatment showed values ND (Figure 4(a)).

3.4.2. IL-10. IL-10 differed significantly before and during treatments ($F_{[1,10]} = 63.6$, $P < 0.0001$). This cytokine showed increase (SSRIs = 633 ± 84 and SSRIs plus hDLEs = 812 ± 100) at W5, W20 (SSRIs = 513 ± 151 and SSRIs plus hDLEs = 849±65) versus W0 (SSRIs = 766±84 and SSRIs plus hDLEs = 770±69) and HVS (527±99). In contrast, IL-10 levels declined at W36 (SSRIs = 450 ± 94 and SSRIs plus hDLEs = 697 ± 67) and W52 (SSRIs = 347 ± 31 and SSRIs plus hDLEs = 527 ± 99). At the end of the followup study, IL-10 levels of SSRIs group showed levels comparable with the healthy volunteers (Figure 4(b)).

3.4.3. IL-13. Before treatment (W0), patients have significantly higher IL-13 levels than healthy volunteers (SSRIs = 3725 ± 708 and SSRIs plus hDLEs = 3630 ± 799 versus HVs = 1633 ± 172). IL-13 differed significantly during treatments ($F_{[1,10]} = 115.3$, $P < 0.0001$). Variations in cytokine levels showed at W5 (SSRIs = 3779 ± 380 and SSRIs plus hDLEs = 4437±505), W20 (SSRIs = 2967±443 and SSRIs plus hDLEs = 3793±579), W36 (SSRIs = 1239 ± 245 and SSRIs plus hDLEs = 2438±57), and W52 (SSRIs = 1116±268 and SSRIs plus hDLEs = 1953 ± 271) versus W0 (SSRIs = 3725 ± 708 and SSRIs plus hDLEs = 3630 ± 799) (Figure 4(c)).

4. Discussion

4.1. Cortisol. Hyperactivity of the HPA axis in response to increased stress is linked to dysregulation of cortisol and serotonin secretion in various psychiatric disorders, such as major depression disorder [7, 28]. Cortisol levels are regulated by a negative feedback system of glucocorticoid receptors (GRs). GR is a steroid-activated nuclear receptor that, on binding to cortisol, translocates to the nucleus, where it targets genes that mediate cortisol and cytokine secretion and neuronal metabolism and plasticity [29].

Hypercortisolemia is common in MDD patients—clinical assays have reported higher cortisol levels in saliva, plasma, and cerebrospinal fluid in depressed patients who have not received pharmacological treatment [30, 31]. Before being administered SSRIs and hDLEs, our patients also experienced hypercortisolemia. Failure of the HPA axis to regulate circulating cortisol levels has been suggested to affect desensitization to GRs during stress and the inability to return to resting conditions, prolonging GR activation and its downstream effects [28].

These findings could explain the hypercortisolemia in patients with MDD, a common comorbidity in this disorder that is regarded as a marker of axis hyperactivity HPA [28]. Further, corticosteroids have a significant function in the link between stress and mood alterations, interacting with serotonin receptors (5-HRT_{1A}, $5HT_2$) [32–34].

SSRIs, the most widely used antidepressants, upregulate extracellular serotonin concentrations by acutely blocking the serotonin transporter 5-HTT. 5-HTT regulates extracellular serotonin concentrations by removing 5-HT from the synaptic cleft [29]. Various clinical studies, including ours, have reported a decline in cortisol levels in fluids of MDD patients who have been treated with SSRIs [20, 35, 36]. After 1 year of treatment, however, cortisol levels are not comparable to healthy subjects [20].

This study examined the ability of coadministration of hDLEs and SSRI in MDD patients to restore cortisol and cytokine imbalances versus SSRIs alone. Our data show that, in depressed patients who were treated with SSRIs plus hDLE, cortisol levels, fell (54%) from W20 to the end of treatment. In contrast, SSRIs alone decreased such levels from W36 to W52. Notably, those who were given SSRIs plus hDLEs showed cortisol levels more nearby to healthy volunteers.

The underlying molecular mechanism by which SSRIs function is unknown. 5-HTT controls the reuptake of serotonin from the synapse, and its inhibition by SSRIs increases serotonin levels at the synapse. However, SSRIs may influence the endocrine and immune systems of depressed patients. *In vitro* studies have demonstrated that SSRIs enhance GR-mediated transcription in the presence of cortisol [37] and

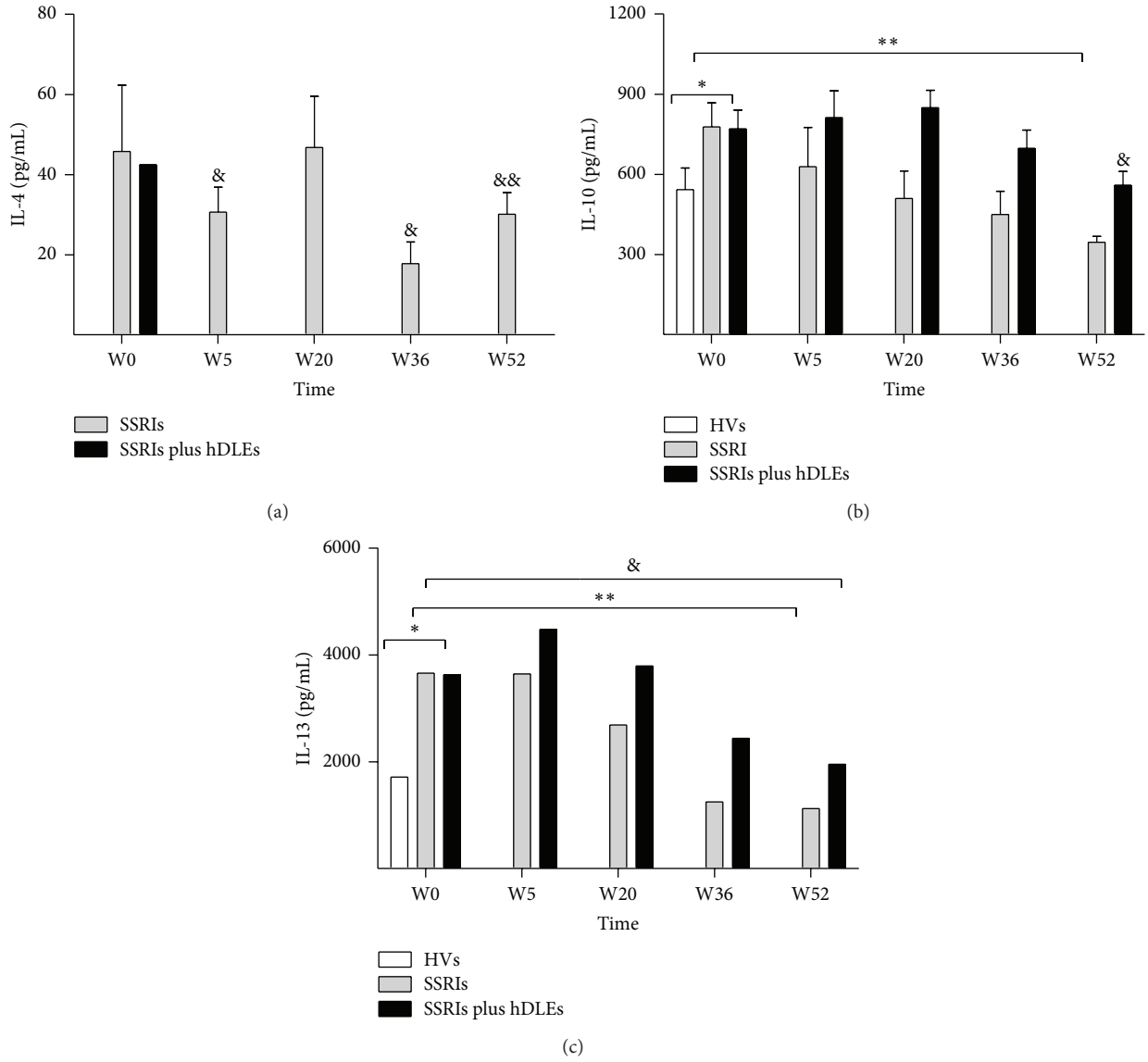

Figure 4: Serum anti-inflammatory cytokines detected by capture ELISSA assay in HVs and MDD patients. Patients were treated with SSRIs or SSRIs plus hDLEs during 52 weeks of study. The statistical analyses were as follows. First, the patient group before antidepressant treatment (W0) was compared with the control group (HVs). Second, the values before the antidepressant treatments (W0) were compared with those during the treatment (W5, W20, W36, or W52) in patients. Third, the data of patients at W52 versus HVs were compared. IL-4 (a), IL-10(b), and IL-13 (c). HVs were not detectable. IL-4: $^{\&}P < 0.0001$ SSRIs treatment at (W) 5 and 36 versus W0. $^{\&\&}P < 0.001$ between SSRIs treatment at W52 versus W0. IL-10 and IL-13: $^{*}P < 0.0001$, significant difference before treatments versus HVs. IL-10: $^{**}P < 0.001$ between SSRIs treatment at (W) 5, 20, and 36 versus W0. $^{\&}P < 0.0001$ between SSRIs plus hDLEs treatment at W52 versus HVs. IL-13: $^{**}P < 0.001$ between SSRIs treatment at (W) 5, 20, and 36 versus W0. $^{\&}P < 0.0001$ between SSRIs plus hDLEs treatment at (W) 5, 20, 36, and 52 versus W0. Data are expressed as mean ± SD. HVs: healthy volunteers; MDD: major depression disorder; W: weeks.

have proposed that antidepressants inhibit membrane-bound steroid transporters, increasing the intracellular concentrations of the glucocorticoids [18], in turn enhancing GR expression and function and restoring the negative feedback by cortisol [38, 39].

Serotonin stimulates the secretory activity of the adrenal glands through 5-HT4 receptors [40, 41]. Moreover, glucocorticoid receptor antagonists increase the response to SSRIs with elevated serotonin levels [42, 43]; in contrast, exogenous administration of SSRIs decreases cortisol secretion *in vitro* [44]. The early and significant decline in cortisol levels in MDD patients who have been treated with SSRIs and hDLEs suggests that this combination enhances the effect of SSRIs on cortisol levels.

4.2. Pro- and Anti-Inflammatory Cytokines. SSRIs also target cells of the immune system. A wide range of cytokine-producing cells constitutively express cortisol and the serotonin receptors 5-$HT_{1A,}$ 5-$HT_{2A,}$ 5-$HT_{1B,}$ and 5-HT_3 [29, 45, 46]. Serotonin receptors activate cAMP-dependent pathways

and mediate synthesis and release of cytokines and cellular proliferation [9]. Cortisol modulates cytokine gene transcription and lymphocyte proliferation. Studies of dexamethasone have reported alterations in GR in leukocytes of depressed patients, such as decreased nuclear translocation [47] and cellular proliferation [48]. In addition, lymphocytes from MDD patients have a lower density of 5-HTT [49] and impaired 5-HT_{1A} receptor functions [45, 46].

Variations in cortisol and serotonin concentrations can directly affect cytokine-producing cells and modulate the pattern of cytokine release [50]. We have reported an anti-inflammatory cytokine profile in MDD patients before pharmacological treatment [20, 21]. In this study, MDD patients had significantly higher IL-4, IL-10, and IL-13 levels at W0 versus healthy volunteers and lower proinflammatory cytokine levels (IL-1β, IFN-γ, and IL-2).

The immune system responds to stressful stimuli by secreting proinflammatory cytokines [51], but when the rise in stress becomes chronic, the cytokines that are produced by immune cells activate the HPA axis, stimulating the adrenal cortex to synthesize and release glucocorticoids, which ultimately suppress proinflammatory gene expression [34]. For example, glucocorticoids upregulate IL-4, IL-10, and IL-13 production and can induce immunosuppression with higher and sustained glucocorticoid secretion.

Previous studies are consistent with our data, in which clinical assays have shown changes in the balance of pro- and anti-inflammatory cytokines in patients with MDD without pharmacological treatment [21] and lower IFN-γ and IL-2 levels [52]. Moreover, suicidal depressed patients have a proinflammatory cytokine profile, whereas no such patients have an anti-inflammatory profile [53]; in our study, an exclusion criterion was the presence of suicidal ideation. Notably, *in vitro* studies have reported that high levels of anti-inflammatory cytokines, such as IL-4, are associated with elevated cortisol and can impede the capture of 5-HT [32], significantly downregulating cytokines, such as IL-2 and IFN-γ, in MDD patients [21], acting primarily through immunological antagonism between pro- and anti-inflammatory cytokines.

Antidepressants increased serotonin levels in MDD patients, inducing clinical remission at W20, as assessed by HDRS and BDI scores. At this time point, IL-2 and IFN-γ levels rose significantly compared with W0 although there were no substantial changes in cortisol levels between SSRIs alone and SSRIs plus hDLEs, which increased the levels of these cytokines and decreased cortisol levels; this reduction was maintained until the end of treatment. The rise in proinflammatory cytokines might be attributed to the immunostimulatory action of serotonin. Studies have demonstrated that the effects of serotonin on the immune response are dose dependent—proinflammatory cytokine secretion and cellular proliferation occur at physiological serotonin concentrations (0.15 to 1.5 μg/mL of serotonin), whereas such secretion declines at supraphysiological doses (15 μg/mL) [50, 54].

These findings contrast with other reports, in which SSRIs decreased proinflammatory cytokine levels in MDD patients [55], which might be due to the time of administration of SSRIs (our study followed up for 52 weeks versus 6 weeks in other studies), the patient demographics (gender, ethnic group, and family history), or depression subtypes [56]. Throughout the administration of SSRIs and SSRIs plus hDLEs, proinflammatory cytokine levels differed in MDD patients. At W20, the SSRI group and SSRI plus hDLE group experienced the largest increase of IFN-γ and IL-1β; whereas the latter maintained such levels to W52, the levels in the SSRIs group were comparable with those of healthy volunteers.

In contrast, IL-1β levels in the SSRIs group remained higher until the end of treatment, and IL-1β decreased, approximating levels in healthy volunteers. IL-2 levels climbed in the SSRI and SSRI plus hDLE groups until W36, which remained elevated until the end of treatment. At W52, the SSRI group had lower IL-1β levels versus at W0.

As discussed, SSRIs increase circulating levels of plasma serotonin [35], the physiological doses of which can upregulate IL-1β and IFN-γ secretion [50]. This evidence could explain the rise in proinflammatory cytokines in both patient groups.

DLEs have immunomodulatory and immunostimulatory effects in various infectious diseases, autoimmune diseases, and cancers [57–59]. The coadministration of SSRIs and hDLEs increased IL-1β, IL-2, and IFN-γ levels. In addition, *in vitro* studies have shown that hDLEs induce IFN-γ secretion in Jurkat cells [23]. The mechanism underlying the effects of hEDL still not elucidate, but certain peptides are recognized by innate immune receptors, such as TLRs, on macrophages, B cells, and dendritic cells [60, 61]. These antigen-presenting cells might present hEDL peptides to T cells and induce the release of proinflammatory cytokines.

In our study, MDD patients had an anti-inflammatory cytokine profile that was associated with increased cortisol levels before treatment. Healthy volunteers had undetectable levels of IL-4. SSRIs and SSRIs plus hDLEs downregulated IL-10 and IL-13, but SSRIs failed to reduce the increased levels of IL-4. Notably, *in vitro* studies have linked high levels of anti-inflammatory cytokines, such as IL-4, to elevated cortisol, which can inhibit the capture of 5-HT [32], causing a significant decrease in cytokines, such as IL-2 and IFN-γ, in MDD patients. In the SSRI-plus-hDLE group, IL-4 levels were not detected during clinical followup.

At W52, patients who were given SSRIs and hDLEs experienced a decline in IL-10 and IL-13 to comparable levels in healthy volunteers. In contrast, SSRI-treated patients showed significant variations in IL-10 and IL-13 levels versus healthy volunteers at W52.

Limitations. The limitations of our study are the small sample size and open-label study design without placebo control group. In addition the total scores of the Hamilton Depression Rating Scale (HDRS) showed no significant changes between treatments but will be necessary to analyze if the assessment of each "symptom cluster of HDRS" (anxiety, affective, or somatic symptoms) is associated to molecular variations detected in this study.

5. Conclusions

Our report is the first study to analyze the balance between pro- and anti-inflammatory cytokines over 52 weeks of treatment, using an alternative treatment to the classical pharmacologic regimen of SSRIs. hDLEs potentiate the effects of SSRIs on HPA axis hyperactivity by decreasing cortisol levels early in the course of treatment; at the end of the study, patients who were treated with SSRIs and hDLEs consistently had a mixed pro- and anti-inflammatory cytokine pattern. Further studies with more MDD patients are necessary to determine the significance of these findings and their clinical implications for the development of alternative therapeutic approaches in the treatment of major depression.

Conflict of Interests

All authors disclose that none has a commercial association that might pose a conflict of interests in connection with this paper.

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Research Article

Potential Immune Modularly Role of Glycine in Oral Gingival Inflammation

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Gingival epithelial cells (GECs) represent a physical barrier against bacteria and are involved in the processes of innate immunity. Recently, an anti-inflammatory and immune-modulatory effect of the amino acid glycine has been demonstrated. However, there is only little information about the immune-modulatory effects of glycine in oral tissues. This study aimed to investigate the existence and role of the glycine receptor in gingival tissue analyzing tissues/cells from extracted human molars via immunohistochemical analysis. *In vitro*, GECs were challenged by inflammatory conditions with IL-1β alone or in combination with glycine and analyzed for cytokine expression of IL6/IL8 via real-time PCR. On protein level, the effect of nuclear translocalization of NFκB protein p65 was analyzed using immunofluorescence analysis. A distinct proof of the GlyR in oral gingival tissue and keratinocytes could be demonstrated. Isolated challenge of the keratinocytes with IL-1β as well as with glycine resulted in an upregulation of IL6 and IL8 mRNA expression and activation of NFκB pathway. The presence of glycine in combination with the inflammatory stimulus led to a significant decrease in inflammatory parameters. These results indicate a possible anti-inflammatory role of glycine in gingival inflammation and encourage further research on the utility of glycine in the prevention or therapy of inflammatory periodontitis.

1. Introduction

Periodontitis typically starts with inflammation of the gingiva and proceeds by spreading into the deeper structures of the periodontium, leading to progressive destruction of periodontal tissues and the alveolar bone and to the loss of teeth [1]. As a major part of the gingival tissue, gingival keratinocytes represent a physical barrier to infections by periodontal pathogens [2]. While the epithelium was previously thought to provide only a passive role in inflammation, recent articles demonstrated a new perspective to inflammatory conditions assigning an active role to the epithelium in the host response to bacterial infections [3]. According to these authors, the epithelium reacts to bacterial challenges by signaling host defense and integrating innate and acquired immune responses.

Signaling pathways of gingival keratinocytes that are modulated by bacteria such as *Porphyromonas gingivalis* in the course of periodontal infection include changes in intracellular calcium ion (Ca^{2+}) concentrations [4, 5]. Izutzu et al. [4] demonstrated that *P. gingivalis* invasion and inflammatory response in human gingival epithelial cells is related to a release of Ca^{2+} from intracellular reservoirs and subsequent increase in cytosolic Ca^{2+}. Contact between *P. gingivalis* and epithelial cells is shown to activate the host-cell Ca^{2+} signaling system, a further sign for its inflammatory impact.

Similar inflammatory signaling is also reported for the cytokine interleukin 1β (IL-1β) which is involved in the pathogenesis of many inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, and also periodontitis [6–8]. In human keratinocytes, induction of IL-1β was demonstrated to take place upon

stimulation with bacterial lipopolysaccharide (LPS), physical or thermal injury, ultraviolet irradiation, and a variety of cytokines, that is, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), interleukin 6 (IL6), transforming growth factor-β (TGF-α), and IL-1β [6, 9]. IL-1β is reported to trigger chemotaxis of neutrophil granulocytes as well as T and B cell activation. In addition, it stimulates the expression of the early response genes, multiple cytokines, and inflammatory factors that drive extracellular matrix degradation [6, 7].

For an anti-inflammatory purpose, the use of glycine is reported to induce beneficial immune-modulatory and cytoprotective effects [10–12]. L-glycine is the smallest nonessential amino acid that consists of a methylene carbon molecule attached to an amino- and a carboxyl group. In nonnervous tissue, glycine is considered to be biologically neutral. In the last years, however, numerous investigations have revealed significant effects of glycine on the activation of cells belonging to the innate as well as the adaptive immune system, including macrophages, polymorphonuclear neutrophils (PMNs), and lymphocytes [13–15]. In patient's treatment, glycine has been reported to have several beneficial effects including protection against toxicity induced by anoxia, oxidative stress, and various toxic agents at the cell, tissue, and whole body levels [10, 12, 16]. For instance, it was demonstrated that a diet enriched with glycine protected against LPS-induced lethality, hypoxia-reperfusion injury after liver transplantation, D-galactosamine-mediated liver injury, and experimental arthritis [10–12, 17].

The glycine receptor (GlyR) is composed of four 48 kDa α-subunits and a 58 kDa β-subunit and comprises a pentameric complex that forms a chloride-selective trans-membrane channel [18]. In support of pharmacological evidence for the existence of GlyR in nonneuronal cells stated above, recent studies provide molecular evidence for the GlyR in nonneuronal cells. A wide variety of cells such as neutrophils, alveolar macrophages, endothelial cells, and Kupffer cells have been shown to contain glycine-gated chloride channels [10–12, 16]. In addition, Denda et al. [19] demonstrated that the existence of the GlyR in epidermal keratinocytes might play a crucial role in cutaneous barrier homeostasis.

Several reports showed that glycine suppresses formation of inflammatory cytokines [10–12]. As stated above, the production of proinflammatory mediators induced by bacterial LPS depends on the increase in intracellular Ca^{2+}, an effect that was demonstrated to be blunted after treatment with glycine. The exact mechanism of how increased intracellular calcium levels are blocked by glycine is not yet completely understood.

In recent investigations, Breivik and coworkers [20] pointed to the anti-inflammatory potential of glycine in oral tissues. In their animal experiments, these authors were able to demonstrate a significant reduction of artificially induced periodontal infection following a specific glycine diet. Substitution of glycine to animal's food prevented animals from severe periodontal breakdown. However, information is missing whether glycine and its receptors may be able to modulate the development of inflammatory oral gingivitis and if they are able to protect oral tissues from severe periodontal disease.

In this context, the first aim of the present study was expression analysis of glycine receptors in gingival tissue and further analysis of the glycine signaling role in oral gingival keratinocytes. We hypothesized that glycine receptors are also expressed within the oral gingival tissue and that glycine application might be able to modulate the inflammatory response of gingival keratinocytes in inflammatory conditions.

2. Material and Methods

All experimental protocols were reviewed and approved by the Ethics Committee of the University of Bonn.

2.1. Human Gingival Tissue. Tissue samples from human gingiva were obtained during routine extraction treatment of third molars. Human gingiva samples were collected from three different human donors aged between 12 and 14 years showing no clinical signs of gingivitis and periodontitis. The teeth had been extracted for orthodontic reasons and with written parental consent. Following extraction, human teeth including gingiva were perfused with phosphate-buffered saline supplemented with 4% paraformaldehyde for fixation purposes. Afterwards, the gingiva of each tooth was dissected and prepared for light microscopical examination as recently described [21, 22].

2.2. Histology. For histological analyses, all specimens were processed for paraffin histology. To perform histology, 5–7 μm serial sagittal sections of each specimen were prepared. For orientation purposes, selected sections were stained with hematoxylin and eosin.

2.3. Immunohistochemistry. To analyze glycine receptor expression, tissue sections were processed for immunohistochemical detection of glycine receptor expression according to previously established protocols [22, 23]. In brief, sections were incubated with primary antibody of rabbit origin raised against a peptide mapping at the carboxy terminus of the protein (anti-glycine receptor, SYSY, Göttingen, Germany) in a 1 : 250 working solution of TBS/BSA at 4°C overnight in a humidified chamber. A 1 : 100 dilution of a goat anti-rabbit immunoglobulin (Dako A/S, Denmark) was incubated as secondary antibody for 30 min. Following further rinsing, a PAP complex (1 : 150 in TBS/BSA; Dako A/S Denmark) was administered for 30 min prior to the visualization of antibody binding with 3,3′-diaminobenzidine (Sigma Chemicals, USA) solution for about 5 min. Thereafter, specimens were counterstained with Mayer's hematoxylin, dehydrated, and cover-slipped for light microscopical analysis.

In order to prove the specificity of the immunoreactions, negative controls were carried out by (a) omitting the primary antibody or using nonimmune IgG instead and (b) omitting both the primary and secondary antibody and using TBS/BSA instead.

Preadsorption controls were used to exclude unspecific binding of the antibodies to unrelated antigens. The antibody was combined with a twofold excess of blocking peptide and incubated at 4°C overnight. Following neutralization, the antibody/peptide mixture was diluted into the appropriate working solution. Afterwards, immunohistochemistry was carried out as described above.

2.4. Explantation of Gingival Keratinocytes. Gingival keratinocytes/epithelial cells (GECs) were cultured from gingiva of three periodontal healthy patients as described above. The cells were isolated by collagenase digestion and subsequent mechanical isolation of the epithelial layer from the connective tissue as described previously [24]. Following explantation, cells were seeded on 60 mm dishes and cultured in KGM-Medium (Keratinocyte Growth Medium, PromoCell, Heidelberg, Germany) supplemented with $CaCl_2$ (0.15 mM) and 0.5% antibiotics (diluted from a stock solution containing 5,000 U/mL penicillin and 5,000 U/mL streptomycin; Biochrom AG, Germany) under a saturated humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed every other day. Prior to experimental use, gingiva keratinocytes were characterized as described previously [24]. For cell culture experiments, cells were used in passage 4.

2.5. Induction of Gingival Inflammation: In Vitro. To mimic inflammatory impact on gingival keratinocytes similar to the environment in gingivitis, cells were cultured in presence of the amount of 5 ng/mL IL-1β (Promokine, Heidelberg, Germany) as measured in patients suffering from gingival infection and proven to be effective to induce inflammatory response [24–26]. For experiments *in vitro*, cells were grown to a confluent state (90%). One hour prior to stimulation, cells were adapted to a glycine-free medium (SMEM, Supplemented Eagle's Minimum Essential Medium, GIBCO/Invitrogen, Darmstadt, Germany). For stimulation experiments, cells were treated with 5 ng/mL IL-1β in the presence or absence of glycine (5 mM glycine). Following a stimulation time of 30 or 60 minutes, cells were analyzed for changes in gene expression of proinflammatory markers IL6 and IL8. Preliminary experiments demonstrated a 30 min stimulation time for IL6 and 60 min stimulation time for IL8 to be most effective for inflammatory response induction.

2.6. Glycine Stimulation Experiments. To analyze the possible anti-inflammatory effect of glycine on gingiva keratinocytes, in addition to the IL-1β conditioned medium, cells were additionally stimulated with 5 mM glycine (Sigma-Aldrich, Taufkirchen, Germany) as demonstrated to be effective previously by Vardar-Sengul et al. [26]. For negative control, glycine was administered alone.

2.7. Analyses of NFκB Pathway Activation. To further characterize pro- or anti-inflammatory effects of IL-1β with or without glycine, activation of NFκB pathway was investigated. According to a protocol published previously [24], gingival keratinocytes were stimulated for 6 h in combination with IL-1β with and without glycine as described above. After incubation, cells were fixed and further analyzed for nuclear translocation of p65 immune reactions using immune cytochemistry with a primary antibody against NFκB (BioLegend, Uithoorn, Netherlands) in a 1 : 100 concentration [7]. The amount of cells showing positive immunoreactions over the cell nucleus was assessed semiquantitatively by a modification of the grading system published previously [21]. The grading system was set up as follows: 0 = no immune reaction in cell nucleus; 1 = 1/3 of the cell nucleus demonstrated immune reactions; 2 = 2/3 of the cell nucleus demonstrated immune reactions; 3 = immune reaction all over the cell nucleus and the cytoplasm were visible; 4 = immune reactivity was only located in cell nucleus. Cells were counted in relation to total cell number at the investigated region using AxioVision software (Carl Zeiss, Germany). To ensure reproducibility, all counts were performed on four independent experiments per stimulation group.

2.8. RNA Extraction and cDNA Synthesis. RNA was isolated using the "RNeasy Protect Mini Kit" (Qiagen, Hilden, Germany) and quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop, Technologies, Wilmington, USA). 1 μg total RNA was reversely transcribed using "iScript Select cDNA Synthesis Kit" (BioRad, Munich, Germany) with oligo(dT)-primers [24].

2.9. Quantitative Real-Time PCR. Gene expression of β-actin, IL6, and IL8 was analyzed by real-time PCR with the iCycler Thermal Cycler (BioRad). SYBR Green served as fluorophore for online monitoring of generated PCR products as described previously [24]. In brief, all primers were synthesized according to the highest quality standards (Metabion, Martinsried, Germany) and verified by computer analysis for specification (BLAST). Primer sequences are presented in Table 1. Amplification for the detection of β-actin was performed under the following conditions: 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 69°C for 30 s, and 72°C for 30 s. IL6 and IL8 were amplified under the same conditions except for the annealing temperature which was set at 68°C. 50 ng cDNA was added to a master mix containing primers and IQ SYBR Green Supermix (BioRad). The reference gene β-actin was used as standard for normalizing the crossing point. Cloned PCR products derived from the specific primers served as positive controls for the PCR, while water was used as the negative control. PCR was performed for all samples individually. Resulting gene expressions were averaged. Relative differential gene expression was calculated using the method described by Pfaffl [27]. PCR efficiencies were determined with dilution series as listed above (Table 2).

2.10. Immunofluorescence and Cytochemistry. GECs were cultured on sterile coverslips and grown to 90% of confluency. The cells were washed with phosphate-buffered saline (PBS, PAA Laboratories, Cölbe, Germany), fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS, and permeabilized thereafter in 0.1% Triton X-100

Table 1: Primer sequences for real-time PCR for β-actin, IL-6, and IL-8.

Gene	Primer sequence
β-actin	Sense 5′-CATGGATGATGATATCGCCGCG-3′
	Antisense 5′-ACATGATCTGGGTCATCTTCTCG-3′
IL-6	Sense 5′-ATGAACTCCTTCTCCACAAGC-3′
	Antisense 5′-CTACATTTGCCGAAGAGCCC-3′
IL-8	Sense 5′-ATGACTTCCAAGCTGGCCGTGG-3′
	Antisense 5′-TGAATTCTCAGCCCTCTTCAAAAAC-3′

Table 2: Primer efficiencies and corresponding annealing temperatures for β-actin, IL-6, and IL-8.

Gene	Efficiency	Temperature (°C)
β-actin	1.84	69
IL-6	2.12	68
IL-8	2.02	68

in PBS for 10 min. After washing with PBS, nonspecific binding was blocked by a 60 min treatment in a 1% bovine serum albumin solution (BSA; Sigma-Aldrich). Incubation with the primary anti-glycine receptor antibody (SYSY, Göttingen, Germany, 1:250) was performed overnight at room temperature. Following extensive washing, a Cy-3-conjugated anti-rabbit IgG secondary antibody (Dianova, Hamburg, Germany) was applied for 50 min at room temperature. Finally, cells were washed three times with PBS. Nuclear staining was achieved by incubating the cells in DAPI for 8 min, followed by washing twice with Aqua dest. Cells were mounted with Mowiol/DABCO (Roth, Karlsruhe, Germany) for fluorescence microscopic imaging using a Zeiss fluorescence microscope (AXIO Imager A1, Carl Zeiss MicroImaging). For cytochemistry, a horseradish-peroxidase- (HRP-) conjugated secondary antibody (Dako Invision) was used instead and cells were counterstained with hemalaun as described above.

2.11. Statistical Analysis. The PCR data are presented as means ± SD of 10 independent experiments. Statistical significant differences in means were assessed using one-way analysis of variances (ANOVA) in GraphPad Prism version 4.03 for Windows (Graph Pad Software, San Diego, California, USA, http://www.graphpad.com/). A value of $P < 0.05$ was considered statistically significant.

3. Results

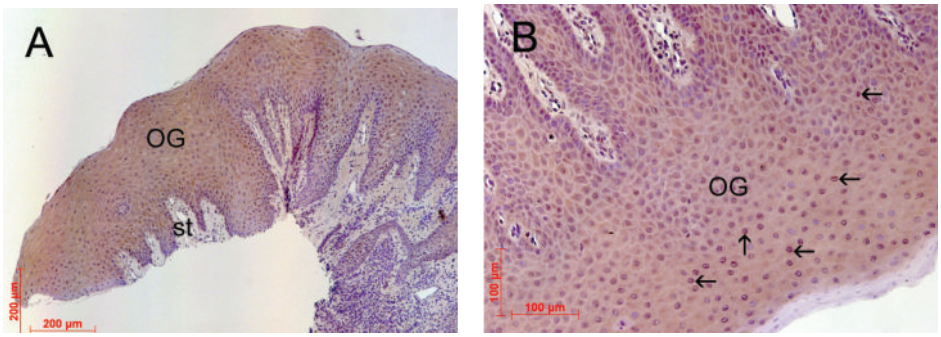
3.1. Presence of the Glycine Receptor in Human Gingival Tissue. Explants of human gingival tissue were analyzed for glycine receptor expression by immunohistochemistry to investigate whether glycine receptor is expressed in human gingival tissue. The immune staining of healthy human gingival tissue sections revealed a strong glycine receptor expression within the epithelial layers of the gingiva tissue. Within the investigated samples, glycine receptor immune reactivity was mainly concentrated to gingival keratinocytes (Figures 1(a) and 1(b)). At cellular level, positive immune reactions were mainly located at both cell membranes and in the cytoplasm of the cells. The underlying basal membrane of the gingiva tissue as well as the other connective tissues showed no positive immune reactions for the glycine receptor.

3.2. Demonstration of Glycine Receptor in Isolated Gingiva Keratinocytes. In a second step, the aim was to verify the observed *in vivo* findings of glycine receptor expression in human gingival tissue sections in isolated human gingival keratinocytes in order to provide the basis for further experiments on those cells. Similar to the *in vivo* findings, positive immune reactions for glycine receptor were also found in cell cultures of isolated gingival keratinocytes. In our experiments fourth passage gingival keratinocytes from healthy humane donors demonstrated a significant amount of immune reactions for glycine receptor expression mainly located on the outer cell membrane and in the cell cytoplasm (Figures 2(a), 2(b), and 2(c)).

3.3. Application of Isolated Challenge with IL-1β. To mimic an inflammatory impact on gingival keratinocytes similar to conditions in oral gingivitis in patients, cells were cultured in presence of the amount of IL-1β which was measured in gingival inflamed patients previously [26]. Following IL-1β stimulation, an impact on mRNA expression of proinflammatory markers interleukin 6 and interleukin 8 was observed. Compared to untreated controls, the mRNA expression of IL6 was increased by 2.3-fold (Figure 3(a)) and IL8 expression was upregulated by 3.5-fold (Figure 3(b)). Changes in IL6 and IL8 were demonstrated to be significant to control cultures.

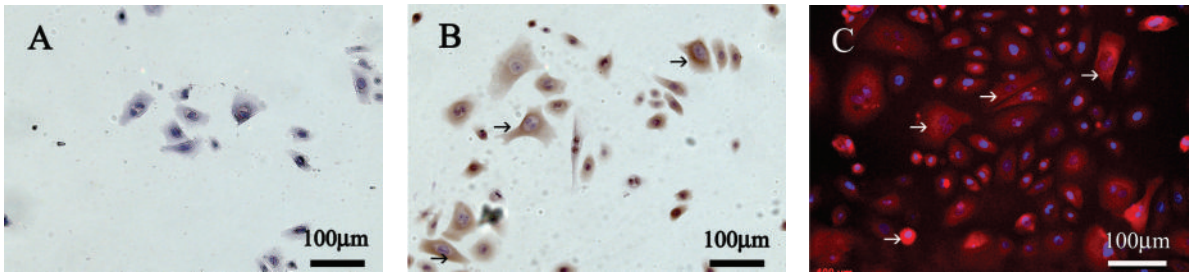
3.4. Effect of Glycine on IL6 and IL8 mRNA Expression. In addition to the IL-1β administration, cells were additionally stimulated with glycine to analyze the anti-inflammatory potential of glycine on induced gingival inflammatory conditions. Following glycine administration, the expression of the analyzed proinflammatory markers was demonstrated to be downregulated. The observed inflammatory effect on IL6 expression was significantly reduced compared to the isolated IL-1β stimulation group (0.8- instead of 2.3-fold of control; Figure 3(a)). The effect on IL8 mRNA expression was also significantly reduced when glycine was added to the conditioned cell culture medium (2.4- instead of 3.5-fold of control (Figure 3(b)). Interestingly, isolated application of glycine seems also to induce a slight upregulation of IL6 in mRNA expression (Figure 3(a)). In combination with IL-1β a downregulation was observed.

3.5. Effect of Glycine on NFκB Pathway-Mediated Inflammatory Response. As a second approach, the immune-modulatory effect of glycine on inflammatory signaling pathway NFκB using the NFκB related protein p65 was analyzed. In this context, changes were addressed for p65 protein expression which is localized in the cytoplasm in noninflammatory conditions. In the present experiments, p65 protein was demonstrated to be located in the cell cytoplasm of untreated human gingival keratinocytes (Figure 4).



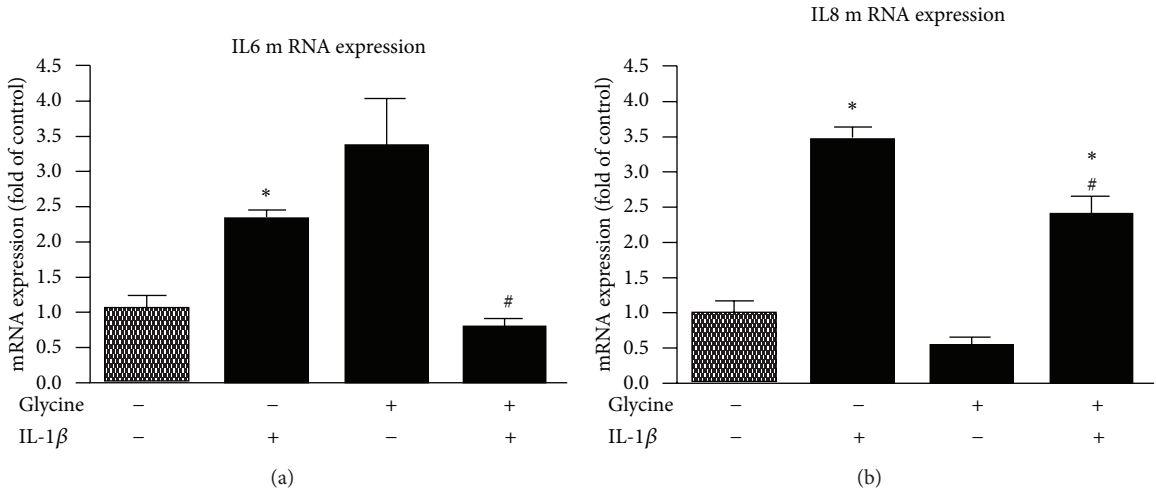
(a) (b)

Figure 1: Demonstration of the glycine receptor (GlyR) in gingival tissue. Overview (a) of oral tissue (magnification ×10) isolated from extracted upper third molars showing different region of the gingival tissue (OG: oral gingival tissue; st: subepithelial tissue). (b) Representative gingival tissue section (magnification ×200) assessed by immunohistochemistry using an anti-glycine receptor antibody (SYSY, Göttingen, Germany) at 4°C overnight and counterstaining with DAB (brown color). There was strong immune reaction in the epithelial layers of the gingiva, namely, in gingival keratinocytes. The underlying basal membrane as well as the connective tissue showed negative immune staining.



(a) (b) (c)

Figure 2: Cytochemical ((a): negative control, (b): anti-glycine receptor) and immunofluorescence ((c) anti-glycine receptor) staining for Glycine receptor in cultured human gingival keratinocytes (red color, black arrow). For nuclear staining, cells were treated with DAPI (blue color, (c)).



(a) (b)

Figure 3: Effect of IL-1β and glycine treatment on the mRNA expression of the proinflammatory cytokines IL6 (a) and IL8 (b). Confluent cultures of gingival keratinocytes were treated with IL-1β either alone or in combination with glycine to induce inflammatory conditions *in vitro*. Glycine administration to inflammatory challenged gingival keratinocytes resulted in a decreased proinflammatory cytokine mRNA expression of both IL6 (a) and IL8 (b). Results are presented as means ± SD ($n = 10$); $^{*}P < 0.05$ difference between groups following ANOVA analysis.

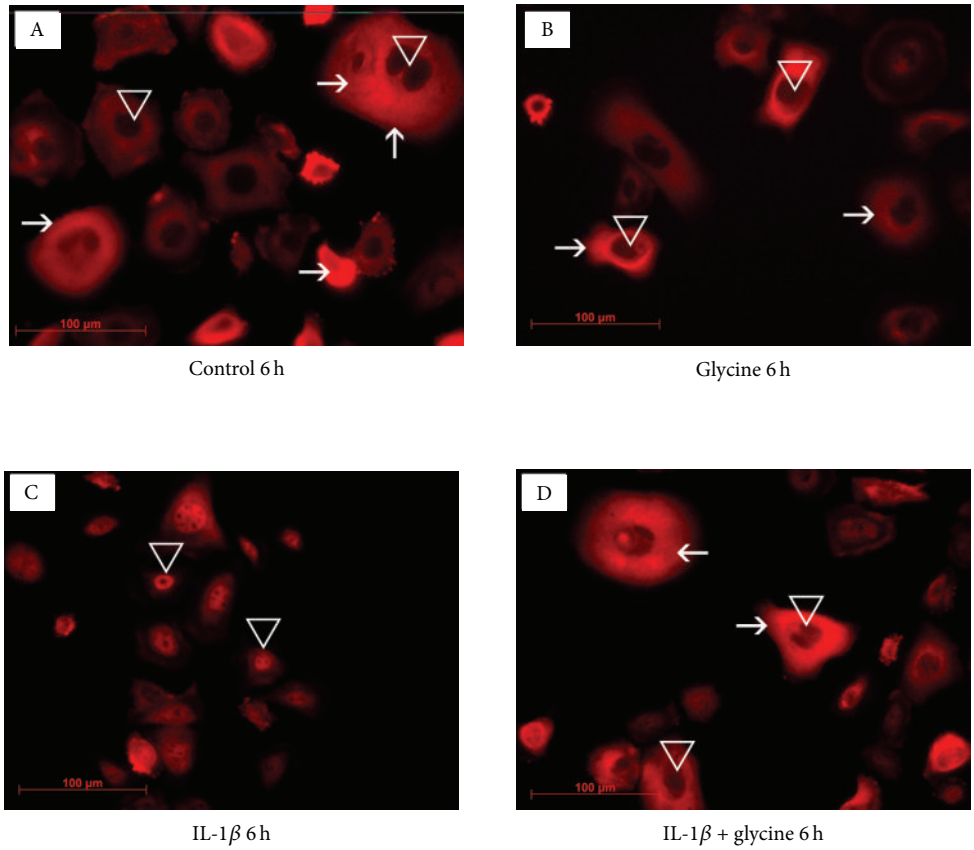


Figure 4: Effects of IL-1β and/or glycine on NFκB signaling. (a) Immune fluorescence staining of NFκB signaling protein p65 of cells (A), glycine (B), IL-1β (C), and IL-1β + glycine (D) treated human gingival keratinocytes. The photographs demonstrate a localization of p65 protein primarily in the cell cytoplasm in untreated cells. Untreated controls (A) and glycine treated cells (B) did not affect p65 expression. Following the induction of inflammation by IL-1β administration, NFκB pathway was activated as demonstrated by translocation of p65 protein to the cell nucleus (C). The addition of glycine to the inflammatory cell culture medium reduced the observed p65 protein translocation (D). (b) Semiquantitative assessment of p65 protein translocation in human gingival keratinocytes following administration of IL-1β in combination with and without glycine. $^{*}P < 0.05$ difference between treated and control groups following ANOVA analysis; $^{\#}P < 0.05$ difference between IL-1β and IL-1β + glycine treated groups.

Following the induction of inflammation by stimulation with IL-1β, a pronounced accumulation of NFκB in the cell nucleus was observed. When glycine was added to the conditioned cell culture medium the inflammatory translocation of p65 NFκB pathway protein was almost completely abolished. Similar to control experiments, p65 immune reactions were mainly located in the cytoplasm of the cells and did not translocate to the cell nucleus in glycine treated cell culture experiments.

4. Discussion

To our knowledge, this study is the first which analyzes the existence of the glycine receptor in gingival tissue and gingival derived keratinocytes. Both, tissue sections of human gingival tissue and human gingival keratinocytes demonstrated a ubiquitous expression of the glycine receptor in the gingival tissue which was mainly located at the cell membrane and in the cytoplasmic region. Similar to present findings, other authors documented similar findings on the location of the glycine binding receptor (GlyR) in different cell systems. For example, Webb and Lynch demonstrated in their studies on neural cells that the glycine receptor was located on the cell membrane which acts as a receptor with transmembrane domains and extracellular binding sites as well as chloride-sensitive transmembrane channels [28]. Similar to this report, Zhong et al. [11] also demonstrated the GlyR as a membrane receptor in "Kupffer" cells. On the other hand, Béchade [29] observed both a membrane and a more cytoplasmatic localization of the GlyR in oligodendrocytes and in glia cells. From the present findings the expression of GlyR both in the membrane and within the cell cytoplasm in gingival keratinocytes can be suggested.

To mimic the clinical situation of gingival inflammation such as in conditions of oral gingival inflammation in the present *in vitro* experiments, isolated gingival keratinocytes were challenged with IL-1β at the amount being found to be expressed in patients suffering from gingivitis [30]. The used amount of interleukin 1β application has been proven to be effective in the present findings as shown by the upregulation of cytokine mRNA expression levels of IL6 and IL8 and the induction of NFκB activation in treated gingival keratinocytes. Steinberg et al. [7] and McKay and Cidlowski [31] also reported similar effects of IL-1β-induced inflammation in their experiments on gingival keratinocytes after stimulation with IL-1β. Also the inflammatory response of oral cells to cytokine application on protein level of the investigated inflammatory parameters was proven in previous experiments suggesting the effectiveness of the present protocol [24, 32, 33]. A significant inflammatory response of treated specimens was observed in this study and also in reports from other authors. Corresponding to the presented regulation of IL6 and IL8 mRNA expression, also Kraus and coworkers [24] presented in a recent work that bacterial LPS stimulation was able to show similar and even stronger inflammatory responses of gingival keratinocytes as documented in the present study. Again the present data on IL-1β induced inflammation underline the efficiency and capability of IL-β to induce inflammatory reactions.

The addition of glycine to the cell culture medium of inflammatory challenged gingival keratinocytes demonstrated the ability to attenuate the observed inflammatory response of gingival keratinocytes. Since the standard Keratinocyte Growth Medium (KGM) contains different amino acids including glycine, we therefore analyzed its impact on cell culture conditions by using also glycine-free medium in preliminary unpublished experiments. Induction of different inflammatory cytokines in keratinocytes was demonstrated to be nearly identical compared to the used standard medium in these experiments. Therefore the described standard cell culture medium was used in present experiments.

Interestingly an initial upregulation of the mRNA expression of the investigated early inflammatory marker IL6, but not IL8, after 30 min of stimulation with glycine alone was observed. A possible explanation for this regulation might be due to an early cellular response to changes within the cell culture medium by the application of glycine. Interestingly in combination with IL-1β protein this upregulation was inhibited. The regulation of IL8 expression, which is known as a more stable inflammatory marker, was not affected by glycine application. After adding glycine to the cytokine contaminated medium, a significant inhibition of the immediate upregulation of the cytokines IL6 and IL8 at the transcriptional level and a decrease in p65 nuclear translocation were observed. This glycine-induced anti-inflammatory response goes hand in hand with reports of others on observations in several different cell and organ systems. In his recent review article Zhong and coworkers [11] pointed to the observed anti-inflammatory potential of glycine. They reported that glycine is found to be protective under several inflammatory conditions such as shock, endotoxin, and sepsis and to prevent ischemia-reperfusion injury to a variety of tissues and organs including liver, kidney, heart, intestine, and skeletal muscle. Furthermore, glycine also protected against peptidoglycan polysaccharide-induced arthritis and protects the gastric mucosa against chemical and stress-induced ulcers. In their review these authors concluded that glycine appears to exert several protective effects, including anti-inflammatory, immune-modulatory, and direct cytoprotective actions [11]. Furthermore it has been suggested that glycine is also able to act on inflammatory cells such as macrophages to suppress activation of inflammatory transcription factors and the formation of free radicals and inflammatory cytokines. In the light of the findings in the present investigation and the reported effects of glycine in the literature, it can be suggested that also gingival tissue with special regard to gingival keratinocytes is able to perform a glycine-mediated anti-inflammatory response under inflammatory circumstances.

5. Conclusions

In conclusion, the present data demonstrate at first that the glycine receptor is expressed in gingival tissue and indicates an immune-regulatory role for glycine in the response of gingival keratinocytes to inflammatory conditions. Together with recent reports about glycine physiology, the modulatory potential of glycine within the inflammatory process

in gingival tissue becomes obvious. The mapping of intercellular cytokine signaling networks and its modulating reagents, with special regard to glycine that functionally couples gingival tissues, and its activation to induce immune response may also provide information for future potential clinical targets. This could include the prevention of severe gingivitis and the further development of periodontal disease which is discussed to be the result of long lasting gingival inflammation. Furthermore, the immune-modulatory role of glycine in the course of gingival inflammation extends the well accepted knowledge of anti-inflammatory effects of glycine in the oral gingival tissue that again points to glycine as a promising treatment agent in inflammatory conditions.

Authors' Contribution

Teresa Schaumann and Dominik Kraus contributed equally to this work.

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Review Article

Influence of the Cholinergic System on the Immune Response of Teleost Fishes: Potential Model in Biomedical Research

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Fishes are the phylogenetically oldest vertebrate group, which includes more than one-half of the vertebrates on the planet; additionally, many species have ecological and economic importance. Fish are the first evolved group of organisms with adaptive immune mechanisms; consequently, they are an important link in the evolution of the immune system, thus a potential model for understanding the mechanisms of immunoregulation. Currently, the influence of the neurotransmitter acetylcholine (ACh) on the cells of the immune system is widely studied in mammalian models, which have provided evidence on ACh production by immune cells (the noncholinergic neuronal system); however, these neuroimmunomodulation mechanisms in fish and lower vertebrates are poorly studied. Therefore, the objective of this review paper was to analyze the influence of the cholinergic system on the immune response of teleost fish, which could provide information concerning the possibility of bidirectional communication between the nervous and immune systems in these organisms and provide data for a better understanding of basic issues in neuroimmunology in lower vertebrates, such as bony fishes. Thus, the use of fish as a model in biomedical research may contribute to a better understanding of human diseases and diseases in other animals.

1. Immune System in Teleost Fishes

Fishes are the phylogenetically oldest vertebrate group and appeared >560 million years ago. This group includes >27,000 species, representing more than one half of the vertebrates on the planet. The vast majority of fishes are teleosts (teleostei, possessing a bony skeleton) and some are noted for their ecological and economic importance, whereas other species are widely used as biological models for genomic studies and developmental biology [1, 2]. In addition, because these organisms are the first that present adaptive immune mechanisms (Figure 1), the Big Bang of Immunology [3], the study of the immune system of these organisms is of great relevance because it provides information on the evolution of the immune system in vertebrates, thus supporting the understanding of basic aspects of immunology, therefore the possible treatment of emerging diseases in humans and in other animals [4].

1.1. Lymphoid Organs. Fishes, unlike mammals, lack lymph nodes and bone marrow [5]. However, the anterior kidney or pronephros, analog evolutionary of the bone marrow, possesses important hematopoietic functions (precursor hematopoietic cells appear after 96 h as postfertilized (hpf) in mesonephric tubules) and also presents similar functions to those of the adrenal gland of mammals, which is key in connections among the neuroimmune-endocrine systems [6–8]. Additionally, the spleen is the main secondary lymphoid organ in fish and presents a significant number of (IgM^+ B) lymphocytes, in addition to participating in the induction of adaptive immune responses, and is important for the elimination of immune complexes [4]. Regarding

	Innate	Adaptative
Humoral	Antimicrobial peptides	Antibodies:
	Lectins	IgM
	Lysozyme	IgD
	Complement	IgT/IgZ
Cellular	Macrophages	B-lymphocytes
	Neutrophils	T-lymphocytes
	Eosinophils	

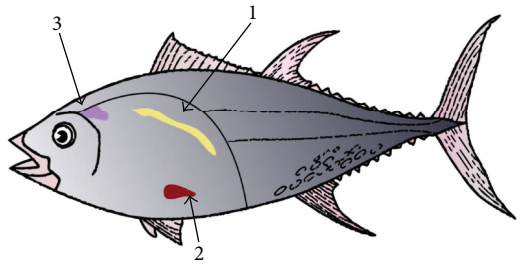

Figure 1: Main humoral, cellular, and anatomical components of the immune system in fishes. Fish lymphoid organs: pronephros (1), spleen (2), and thymus (3).

the thymus, this is a bilobed organ localized in the opercular cavity; it is the major site for T-cell development in fish, as well as in mammals, and presents an involution, except that this phenomenon is greatly influenced by hormonal cycles and environmental changes in the latter [9].

1.2. Innate Immunity. With respect to the mechanisms of innate immunity, these are important in early defense against pathogen infection [10] and also play an instructor role in the induction of the adaptive response [4]. The innate humoral components that are mainly characterized in fishes are antibacterial peptides, lysozymes, lectins, acute-phase proteins, and molecules of the complement system (Figure 1) [11–15].

The cells of the innate immune system are activated by Pathogen recognition receptors (PRR), an important type of these are the Toll-like receptors (TLR). In fishes, it has been described that the majority of TLR are present in mammals (TLR1, TLR2, TLR 3, TLR4, TLR5, TLR7, TLR8, and TLR9). In addition, in fishes the presence of some TLR that has not yet been identified in mammals (TLR18–TLR23) has been described. Additionally, in channel catfish (*Ictalurus punctatus*), the presence has been identified of TLR25 and TLR26, which are apparently present only in fishes [16, 17]. Finally, there are other TLRs, at least eight more, which have been described in other taxa but not in fishes.

The cells of the innate immune response mainly characterized in fishes are the macrophages, neutrophils, and eosinophils [16]. Macrophages are phagocytic cells that are important in early antimicrobial responses; it is been suggested that the phagocytic process in fish is more effective than in murine models when both are compared [18]. Additionally, neutrophils are the first cells to migrate to the site of infection; these cells possess a highly bactericidal capacity through the release of proteolytic enzymes, antimicrobial peptides, and reactive oxygen species (ROS). In addition, the ability that has been recently demonstrated, in fish neutrophils is that of releasing neutrophil extracellular traps (NET), which are complex structures consisting of DNA, histones, and proteins from granules. These structures are responsible for trapping and extracellular killing of bacteria, fungi, parasites, and also for inactivating viruses [19]. Thus, it has been demonstrated that NETs production can be ROS-dependent or -independent. Finally, fish eosinophils, as in mammals, release cytoplasmic granules against extracellular parasites [20, 21].

1.3. Adaptive Immunity. Adaptive immunity mechanisms in fishes play a vital role in protection against recurrent infections through the generation of cellular and humoral mechanisms, which generate immunological memory, mediated by T- and B-lymphocytes and antibodies [22]. Fishes are the first vertebrates in which clonal selection and genetic rearrangement in lymphocyte receptors presents [4].

In fishes, leukocytes have been reported with T-cell activity, similar to that of the T-helper and cytotoxic cells of mammals; also, in some species of fish, some structurally conserved cellular markers are present, including Clusters of differentiation $(CD)3^+$ and T-cell receptors (TCR). Furthermore, based on the profile of cytokines, these also possess T-cell subpopulations similar to those reported in mammals [23].

B-cells are characterized by the expression of antigen receptors (B-cell antigen receptors (BCR)) in the membrane. In teleost fish, B-cells activated, plasmablasts, and plasma cells were identified, which were differentiated from each other mainly by their ability in the production of antibodies [24, 25].

Soluble antibodies that have been identified in fishes are primarily (IgM), which are tetrameric and present in high concentrations in plasma and IgD, which, as in mammals, are expressed on the surface of B-lymphocytes. Other antibody isotypes have been identified in fishes, including IgT and IgZ, which are present mainly in the mucosa, such as in intestine, skin, and gills [26, 27]. Innate and adaptive mechanisms in fishes, similar to those occurring in mammals, are regulated and interconnected by cytokines. Of these, interleukin 1β (IL-β), IL-6 and tumor necrosis factor alpha (TNF-α) have been well characterized in these organisms. Other cytokines reported in fish include IL-2, IL-4, IL-8, IL-10, IL-12, and tumor growth factor beta (TGF-β) [28–30]. In addition to cytokines, the immune system of teleost fishes is regulated by neuroendocrine interactions, primarily through the hypothalamic-pituitary-interrenal (HPI) axis, because the pronephros, in addition to their roles as lymphoid organs, has important endocrine functions. However, there is also evidence of the effect of hypothalamic-pituitary-thyroid

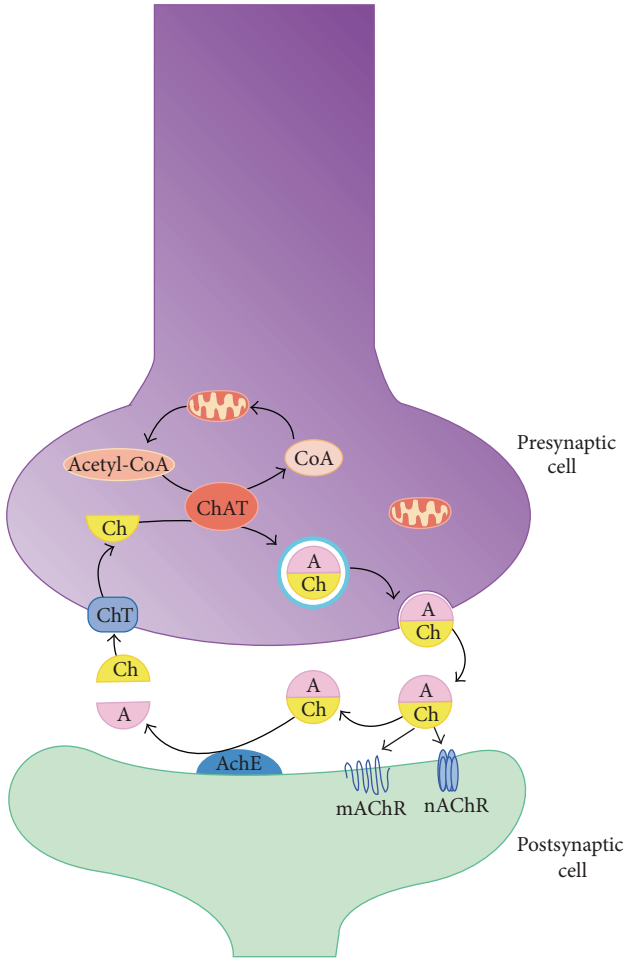

Figure 2: Synthesis of acetylcholine in synapse. A: Acetyl; AChE: acetylcholinesterase; Ch: choline; ChAT: acetylcholine transferase; ChT: choline transporter; CoA: coenzyme A; mAChR: muscarinic receptor; nAChR: nicotinic receptor.

(HPT) and the brain intercommunication-pituitary-gonadal axes on the immune response in teleost fish [31].

2. Cholinergic System in Teleost Fish

Acetylcholine (ACh) is a neurotransmitter that widely distributed in the central and peripheral nervous systems. It is synthesized from choline and acetyl-Coenzyme A (acetyl-CoA) by the enzyme choline acetyltransferase (ChAT) and then is stored in presynaptic vesicles until the cell is activated (Figure 2). When ACh is released into the nerve synapse, this neurotransmitter binds two distinct receptors on the postsynaptic cell: the ionotropic nicotinic acetylcholine receptor (nAChR) and the metabotropic muscarinic acetylcholine receptor (mAChR), which are bonded to G protein. In the synaptic cleft, ACh is hydrolyzed by the enzyme acetylcholinesterase (AChE) into choline and acetate; approximately 50% of the choline hydrolyzed is recovered by the high-affinity presynaptic transporter, which achieves continuous production and releases neurotransmitters [35].

There are evidences that ACh is expressed in bacteria, algae, protozoa, and primitive plants, suggesting an early onset of ACh in evolution. These cells utilize ACh as a neurotransmitter. In fish, the following are some functions that have been linked with this neurotransmitter: visual response of optical circuits; gustatory information processing during feeding, and the processing of motor information [36, 37].

To understand the teleost cholinergic system, it is relevant to identify some important anatomical data about their sympathetic nervous system (SNS), which appears to have particularly unique evolutionary traits for engaging in sophisticated underwater life. This system in teleosts consists of sympathetic ganglia associated with the corresponding spinal nerves, a pair of sympathetic trunks connecting the sympathetic ganglia and the splanchnic nerves. A unique feature of the teleost SNS is that the sympathetic trunks extend into the cranial region and connect several cranial sympathetic ganglia that are associated with the cranial (trigeminal, facial, glossopharyngeal, and vagal) nerves. Organization of the cranial sympathetic ganglia varies among species [38]. In many teleost species, a pair of celiac ganglia is present at the point where the celiac arteries emerge from the aorta. The postganglionic fibers emerging from the celiac ganglia are distributed to the coelomic organs, along with the celiac artery. The celiac ganglia are connected to the sympathetic trunk via the splanchnic nerves.

In mammals, the sympathetic preganglionic neurons (SPN) are clustered in discrete nuclear columns. Developmental studies have demonstrated that SPN, together with the somatic motor neurons, differentiate from a common primitive motor column [39]. In teleosts, the majority of SPN in the sympathetic ganglia appears to be adrenergic; however, a population of ganglion cells (<1%) in the cranial sympathetic ganglia are positive for ChAT; thus, these might be cholinergic [40]. On the other hand, AChE-positive neurons are observed in the periaqueductal gray (PAG) (central gray) in some species of teleost fish, and ChAT-positive neurons were not found dorsally to the central canal but scattered in the lateral region of the central gray [38].

In a comparative study of four fish types, it was observed that distribution of the forebrain cholinergic cells is markedly different among species, suggesting that some structures appeared after the cholinergic system, while in the brainstem, cholinergic structures are well preserved during evolution [41]. The developmental pattern of ChAT-positive neurons has been described in the zebrafish; Arenzana et al. (2005) mentioned that during this fish's development, these neurons are detectable in the forebrain and in the mesencephalic tegmentum region, from 60 hpf, while in the optic tectum, the midbrain does not appear until hatching. In the cerebellum, these cells were observed in the isthmic region and medulla oblongata at the end of the embryonic life. Finally, in the spinal cord, motoneurons are detected from 48 hpf [32].

Several studies have shown that the organization of the cholinergic system in the central nervous system (CNS) is similar among vertebrates; in fishes, however, there is greater variability [42]. Mueller et al. (2004) note the presence of ChAT-positive neuronal cells in different regions of the zebrafish brain, for example, in the telencephalon, preoptic

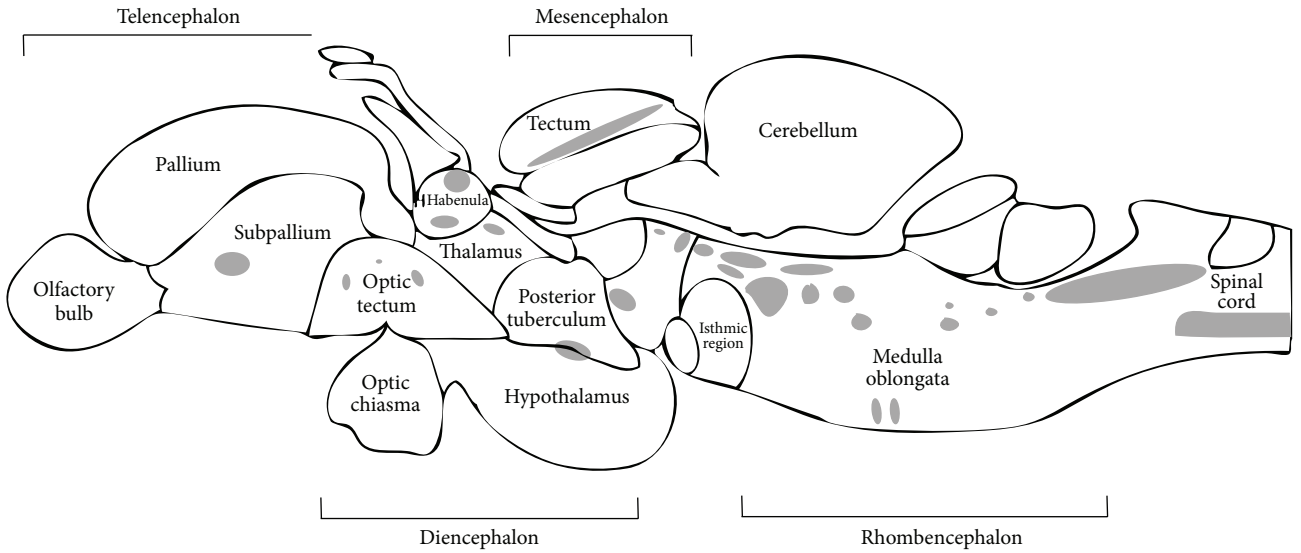

Figure 3: Schematic illustration of location of central nervous cholinergic neuronal populations (gray regions) in adult zebrafish brain (adapted from [32]).

region, diencephalon, mesencephalon, isthmic region, and rhombencephalon [43] (Figure 3).

The nicotinic acetylcholine receptor (AChR) is an integral protein of the postsynaptic membrane that has been studied since the 1970s, first in fishes (as in *Electrophorus* spp. and *Torpedo* spp.) and later in mammals (from mammalian muscle). mAChR are related to neurotransmission, neuromodulation, and olfactory mechanisms, while nAChR are involved in glutamate release and memory construction; both receptors have been characterized in zebrafish [44]. Steele et al. (2007) [45] suggest a role for the mAChR in regulating the heart rate under hypoxia in zebrafish larvae, while the function of the nAChR receptor was elucidated by exposing zebrafish to low doses of nicotine, causing effects on the memory of the fish, in addition to anxiolytic effects, as evidenced by swimming upright [46, 47].

Regarding the characterization of the AChE enzyme, this has been identified in brain tissue of various tropical fish, such as pirarucu (*Arapaima gigas*), cobia (*Rachycentron canadum*), and Nile tilapia (*Oreochromis niloticus*) [48]. Employing immunohistochemical techniques, Clemente et al. (2004) observed AChE-positive neurons in the olfactory bulb and the telencephalon and the diencephalon region remains the least dense in AChE-positive neurons; these were more abundant in the isthmic region and in medulla oblongata subdivisions [37].

3. Cholinergic Influence on the Immune System in Teleost Fish

Currently, the influence of the nervous system on the immune system cells is clear. Thus, there is abundant evidence of the effect of catecholamine, cortisol, and opioids, even serotonin, on the immune response in teleost fish. However, research focused on the study of the effect of the cholinergic system on the immune response of these organisms is very limited [49]. Classical cholinesterase enzymes such as AChE and butyrylcholinesterase (BChE) are sensitive to other neurotransmitters such as serotonin; thus, it could well represent an interface for a crosstalk between these neurotransmitter systems [50, 51].

The two types of cholinesterases (ChE) are present in vertebrates; AChE and BChE exhibit an aryl acylamidase activity (AAA), which is effectively inhibited by cholinergic and serotonergic agents (ACh, specific anticholinesterase drugs and serotonin) [50, 51]. Because the serotoninergic system is involved in pathologies such as anxiety and depression, which in turn influences their immunological responses and cholinergic and that serotonergic drugs sensitively inhibit AAA activity, this could represent a point of crosstalk between the cholinergic and serotoninergic systems. However, due to the complexity of these systems and the lack of precise knowledge that continues to prevail with respect to the brain activity of BChE and also concerning the BChE gene itself that appears to have been lost in some fish lineages [52]. This is why the study of the effect of cholinergic components on neuroimmunomodulation is complex; therefore, addressing a more profound analysis of the relationship between these two systems merits a separate discussion [53].

Although the mechanisms of neuromodulation for the cholinergic system in mammals have been extensively elucidated, investigations of this intercommunication in fish are scarce. In this regard, one of the first reports was conducted by Flory (1990) and Flory and Bayne (1991), who demonstrated, in rainbow trout (*O. mykiss*), that carbachol (a cholinergic agonist) significantly increases the number of antibody-producing cells and the concentration of ROS in leukocytes [54, 55].

Related studies on this fish's spleen structure have shown that this lymphoid organ presents an autonomic innervation.

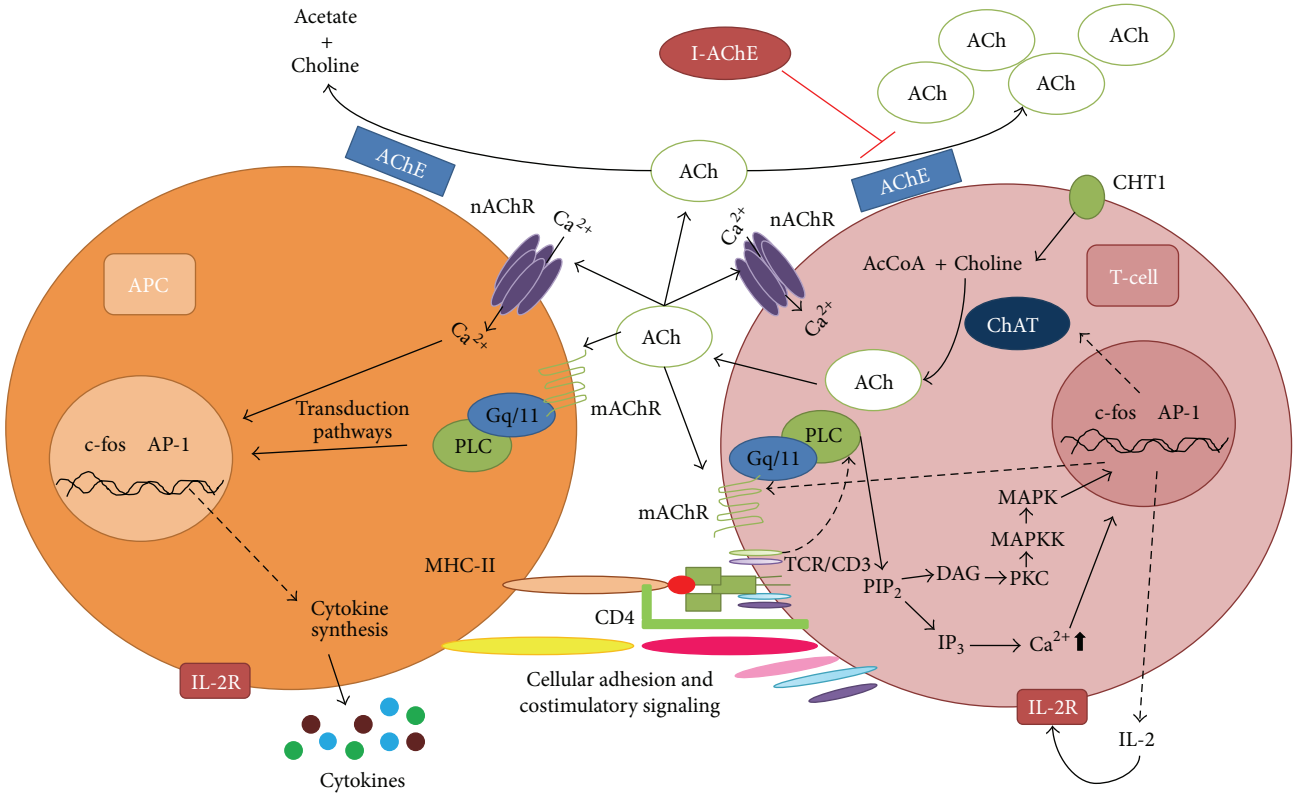

Figure 4: Cholinergic system in cells of the immune system and the effect of acetylcholinesterase inhibitors in mammals. AcCoA: acetyl coenzyme; Ach: acetylcholine; AChE: acetylcholinesterase; AP1: activator protein 1; APC: antigen presenting cell; ChAT: choline acetyltransferase; CHT1: high-affinity choline transporter; DAG: diacyl glycerol; I-AChE: acetylcholinesterase inhibitor; IL-2: interleukin 2; IP_3: inositol-1,4,5-trisphosphate; mAChR: muscarinic ACh receptor; MAPK: mitogen activated protein kinase; MAPKK: MAP kinase kinase; MHC II: major histocompatibility complex class II; nAChR: nicotinic ACh receptor; PIP_2: phosphatidylinositol 4,5-bisphosphate; PKC: protein kinase C; PLC: phospholipase C; TCR: T-cell receptor (adapted from [33, 34]).

Work on the atlantic cod (*Gadus morhua*) has revealed that the teleost spleen receives cholinergic nervous input through a branch of the anterior splenic nerve [56]. Experiments in tench fish or dog fish (*Tinca tinca*) and atlantic cod have shown that exposure to ACh induces a significant reduction of splenic tissue, while exposure to atropine reversed this effect [57].

Moreover, numerous studies have shown that anticholinergic substances (AChE activity inhibitors), such as organophosphorus pesticides (POF), are able to modulate the immune response of fish, leading in the majority of cases to its immunosuppression [33, 34, 58]. Notwithstanding this, the mechanism of immunotoxicity of these compounds remains unclear. Studies by our research group have shown that exposure, *in vivo*, of Nile tilapia (*O. niloticus*) to diazinon (an AChE inhibitor) reduces the proliferative capacity of splenocytes and increases ACh concentration in the spleen, while *in vitro* exposure to this pesticide or to diazoxon (main metabolite of diazinon) does not affect lymphoproliferation. However, lymphocytes exposed to ACh exhibited reduced lymphocyte proliferation [59, 60], suggesting a possible effect of the POF immunotoxicant through alterations in neuroimmunomodulation through cholinergic pathways (Figure 4).

3.1. Nonneuronal Cholinergic System and Lymphocytes. ACh is a major neurotransmitter and its presence has been demonstrated not only in neuronal tissue, but also in prokaryotic and eukaryotic cells, from bacteria to mammalian cells, suggesting the presence of this molecule along evolution [36].

In mammals, the presence has been demonstrated of ACh in extraneuronal tissue, including gastrointestinal epithelium, respiratory, urogenital, placental, and vascular endothelial cells and lymphocytes [61]. ChAT enzyme is constitutively expressed in virtually all cells. In nonneuronal cells, ACh is synthesized and released continuously, in small quantities, into the extracellular environment to maintain cellular homeostasis and to regulate basic cellular functions such as mitosis, differentiation, cytoskeleton organization, and cellular interactions [35].

In terms of immune system cells, it has been demonstrated that these possess, in their membrane, muscarinic (mAChR) and nicotinic (nAChR) receptors, through which it regulates their function [61–64]. Furthermore, ChAT enzyme expression in $CD4^+$ and $CD8^+$ T-cells has been confirmed, suggesting that lymphocytes possess all of the necessary biochemical machinery to produce this neurotransmitter, thereby regulating their functions in an autocrine manner

[65]. Furthermore, *in vitro* studies with human mononuclear cells have reported the presence of concentrations of ACh in 0.3 pmol/10^6 cells, with a synthesis capacity of 2.90 ± 0.84 nmol/mg protein/h [66].

Experimental data obtained by means of *in vitro* models and in the absence of neuronal innervation have shown ChAT production in B cells, macrophages, and dendritic cells from mouse; production of this enzyme appears to be upregulated by TLR activation, a pathway through MyD-88 [65]. Moreover, Neumann et al. in 2007 [66] showed, in human leukocytes, that antagonists of nicotinic and muscarinic receptors (tubocumarin and atropine, resp.) significantly decreased the phagocytic functions of granulocytes, but did not change the migration of these cells, whereas in Jurkat cells (the human helper T-lymphocyte leukemic line), exposure to oxotremorine-M (Oxo-M), a cholinergic agonist, significantly increases the synthesis of IL-2, which could be related with transcriptional factor activator protein-1 (AP-1) and mitogen-activated protein kinases (MAPK) [67], while in experiments in MOLT-3 cells (the human T-cell leukemia line), the involvement has been suggested of the protein kinase C (PKC) signaling pathway-MAPK, cyclic adenosine 3′,5′-monophosphate (cAMP), and calcineurin in the synthesis of ACh [64] (Figure 4).

In general, according to the data obtained in mammalian models, it has been proposed that cholinergic activity increases as a result of direct contact between TCR/CD3 molecules, CD4 and CD8 coreceptors, and other accessory molecules [63]. However, cholinergic component data and extraneuronal cholinergic neuroimmunomodulation mechanisms in fish are scarce.

4. The Cholinergic System in Fish: Another Approach in Biomedicine

The cholinergic system, in addition to its exerting a significant influence on the functioning of the immune system of vertebrates, is also essential for homeostasis of the organism. Cholinergic components are related to physiological changes caused by insecticides, poisons, and chemical weapons, as well as by human degenerative diseases such as myasthenia gravis and Alzheimer's disease (AD) [68].

Therefore, besides the study of cholinergic influence on the immune system of fishes, there is now a growing interest in developing new biological models that permit the study of neuromodulation. Among the species of fish that have been employed in this aspect, we find Pacific electric ray (*Torpedo californica*), eel (*Electrophorus electricus*), goldfish (*Carassius auratus*), Nile tilapia (*O. niloticus*), and zebrafish (*Danio rerio*) [69–72].

Myasthenia gravis is an autoimmune disease in which autoantibodies are generated against a cholinergic receptor. Research utilizing fishes as a model have been prominent in the study of this disease. Some species, such as *T. californica*, have electric organs whose function is dependent on the cholinergic system; thus, these organisms are a rich source of AChR, molecules that have been investigated to determine the epitopes related to the development of this disease [73].

Another disease in which the use of fish is proposed as a study model is AD, most common form of dementia and which is characterized mainly by massive neuronal loss and impaired synaptic processes localized in the cerebral cortex, particularly in the frontal and temporal lobes and the hippocampus. AD is related to cholinergic system dysfunctions, such as the loss of cholinergic neurons in the basal forebrain and the hippocampus. In this regard, the effect of various cholinergic drugs has been evaluated in zebrafish and it has been reported that scopolamine (a cholinergic muscarinic receptor antagonist) impairs both the acquisition of the passive avoidance response and the retention of the learned response and that physostigmine (an acetylcholinesterase inhibitor that blocks the breakdown of the ACh released at the synaptic site) rescues the amnesic effects of scopolamine. Altogether, these findings could facilitate the use of the zebrafish as a model for the study of cholinergic mechanisms underlying learning and memory [74]. Moreover, studies related to the development of memory have been conducted in zebrafish; the data indicate that nicotine affects the memory of these organisms, similar to what has been reported in mammalian models [75]. Thus, this fish can be used to help understand the molecular mechanisms of the cholinergic system's influence on cognitive functions.

Fishes also have been used to evaluate the effects of neurotoxins, such as the case of anatoxin-a, a nicotinic agonist produced by cyanobacteria that blocks cholinergic neurotransmission to compete for the ACh receptor. This toxin can cause death in humans and other animals. Studies on rainbow trout (*O. mykiss*) indicate that exposure to this toxin induces increased AChE and lactate dehydrogenase activity, suggesting that this neurotoxin induces motor impairments and increases the metabolic demand of exposed organisms [76].

The effect of anticholinesterase pesticides has also been extensively studied in fishes. Carbofuran is a pesticide of the carbamates group that is highly toxic to mammals. In humans, this substance causes salivation, abdominal pain, chest tightness, dizziness, vomiting, and seizures. Studies on the common carp (*Cyprinus carpio*) indicate that this pesticide reduces the hatching rate and also induces body deformities, eye pigmentation, pericardial sac enlargement, and changes in fish behavior [77]. Also, carbofuran induces neuroendocrine dysfunctions in spotted snakehead fish (*Channa punctatus*) and abnormalities in the thyroid gland, possibly through an alteration of the hypothalamic-pituitary-thyroid (HPT) axis of the fish [78]. Moreover, immunotoxicity studies of this type of pesticides have shown that teleost fish comprise an excellent model for both basic research and ecotoxicology studies [79]. Studies carried out in our research group have shown that exposure to diazinon induced in Nile tilapia (*O. niloticus*) increased the RB of phagocytic cells and serum IgM concentration, but this pesticide caused a decrease in the proliferative and phagocytic capacity of leukocytes [59, 80], while chlorpyrifos, another anticholinesterase inhibitor, induced phagocytic index reduction in this fish [81]. Thus, the immunotoxic effects of anticholinesterase pesticides in vertebrates may be associated with alterations in neuronal cholinergic and extraneuronal cholinergic pathways.

5. Concluding Remarks

Studies on the communication between the cholinergic and the immune systems in fish are scarce. However, this type of study could generate relevant data to contribute to the understanding of this bidirectional communication that once a full understanding of neuroendocrine control in fish has been achieved, could approach the study of bidirectional communication in evolutive terms, in addition to understanding the importance of the nonneuronal cholinergic system in nonmammalians models. These approaches certainly guarantee a better understanding of basic aspects and eventually allow the proposal of pharmacological alternatives in clinical medicine. Thus, the use of fish as a biomedical research model could contribute to a better understanding of neuroimmunomodulation mechanisms in vertebrates.

Conflict of Interests

There is no conflict of interests, and the authors declare that they have no direct relationship with the previously mentioned commercial entities or any other related.

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Review Article

Therapeutic Potential of Tolerogenic Dendritic Cells in IBD: From Animal Models to Clinical Application

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The gut mucosa undergoes continuous antigenic exposure from food antigens, commensal flora derived ligands, and pathogens. This constant stimulation results in controlled inflammatory responses that are effectively suppressed by multiple factors. This tight regulation, necessary to maintain intestinal homeostasis, is affected during inflammatory bowel diseases (IBD) resulting in altered immune responses to harmless microorganisms. Dendritic cells (DCs) are sentinels of immunity, located in peripheral and lymphoid tissues, which are essential for homeostasis of T cell-dependent immune responses. The expression of a particular set of pathogen recognition receptors allows DCs to initiate immune responses. However, in the absence of danger signals, different DC subsets can induce active tolerance by inducing regulatory T cells (Treg), inhibiting inflammatory T helper cell responses, or both. Interestingly, several protocols to generate clinical grade tolerogenic DC (tol-DCs) *in vitro* have been described, opening the possibility to restore the intestinal homeostasis to bacterial flora by cellular therapy. In this review, we discuss different DC subsets and their role in IBD. Additionally, we will review preclinical studies performed in animal models while describing recent characterization of tol-DCs from Crohn's disease patients for clinical application.

1. Introduction

The gut mucosa is continuously exposed to external food antigens and pathogens and to commensal flora microorganisms, mostly bacteria and fungi. This constant antigenic stimulation results in controlled inflammatory responses that are effectively suppressed by multiple nonimmune and immune factors. The intestinal immune system is capable of distinguishing between invasive organisms and harmless antigens. The host response to the intestinal microbiota can be categorized into three important categories: (1) the intestinal epithelium, which can efficiently modulate immune response by secreting inflammatory mediators, recruiting DCs and presenting antigens to T lymphocytes, (2) the innate immunity, including anatomical barriers, secretory molecules, and cellular components, that initiate the nonspecific immune response, and (3) the adaptive immunity, which is driven by B and T lymphocytes, responsible for antigen specific immune responses. This tight regulation, necessary to maintain intestinal homeostasis, is altered in IBD, resulting in uncontrolled immune responses to harmless microorganisms. Adaptive immunity is the most putative driver of tissue damage seen in IBD patients, although innate immune responses are definitively a prerequisite for the excessive activation of adaptive immunity [1]. Several studies have proposed that the inappropriate activation of DCs may contribute to the pathogenesis of IBD [2].

DCs are the most potent antigen-presenting cells linking innate and adaptive immune responses. Located in peripheral and lymphoid tissues, DCs are sentinels of the immune

system recognizing and translating pathogenic or harmless signals into immunogenic or tolerogenic responses, respectively. DCs are especially well equipped to continuously sample these tissues for the presence of pathogenic microorganisms, and their detection relies on the recognition of conserved molecular structures, known as pathogen-associated-molecular-patterns (PAMPs) via pattern recognition receptors (PRRs). DCs orchestrate adaptive immune responses linking innate recognition of pathogens and driving and polarizing naïve T cells activation. Due to their physiological properties and the availability of clinical grade reagents, DCs have been safely and successfully used in clinical trials aimed at stimulating an efficient immune response against tumors or infectious diseases [3, 4]. However, only a few recent studies have taken advantage of their specific tolerogenic properties to treat Type 1 diabetes [5] and rheumatoid arthritis patients [6]. Both studies have taken place in the last two years; thus, it is still too early to draw any conclusion in relation to their clinical efficacy. The majority of clinical studies to date have been carried out with *ex vivo* generated monocyte-derived DCs taking advantage of their plasticity. Several protocols to generate tol-DC have been described using different agents, including glucocorticoids such as dexamethasone [7, 8], mycophenolic acid [9], vitamin D3 (1α,25-dyhydroxyvitamin D3) [10], retinoic acid (RA), the combination of dexamethasone and vitamin D3 [11, 12], or rhIL-10 [13], which have been used to render DCs resistant to maturation. Therefore, *ex vivo* generated tol-DCs are considered as therapeutic vaccines to reestablish antigen-specific tolerance in autoimmune disorders. The aim of this review is to discuss DC subsets and their role in tolerance induction, preclinical studies in animal models of colitis, and our recent findings on tol-DC generation and characterization in humans for clinical applications in Crohn's disease patients.

2. Human DC Subsets

Several subsets of circulating DCs have been defined in humans based on the lack of expression of typical lineage (CD3, CD19/CD20, CD14, and CD56) negative markers (lin^-) and high levels of MHC class II (HLA-DR) positive cells [14]. Furthermore, a number of positive DC markers have been used to identify different DC subsets. Plasmacytoid DCs ($CD11c^-$) are identified by the expression of BDCA-2 and BDCA-4 plus CD123, whereas myeloid DCs ($CD11c^+$) can be further subdivided in BDCA-1 ($CD1c^+$) and BDCA-3 ($CD141^+$) positive cells. Although evidence suggests the involvement of a particular DC subset in tolerance induction, it is now accepted that different DC subsets participate in immunity or tolerance showing functional maturation and plasticity depending on environmental signals received. However, recent advances have helped to associate the DC subsets to functional specialization. This functional specialization is linked to the different expression between DCs subsets of PRRs like toll-like receptors (TLR), C-type lectins, and the cytoplasmic NOD family proteins, as well as RIG-I and MDA-5 molecules. Whether this functional specialization is linked to different aspects of tolerance induction is an issue to be formally established.

The gastrointestinal immune system is continuously exposed to potent stimuli from commensal bacteria and food. A specialized network of immune cells is organized in the mucosa in order to maintain immunologic tolerance. How DCs regulate immune response in the gut has been deeply investigated, and several DCs subsets and their function have been identified in mice. But whether these subsets are equivalent in humans needs to be further studied (reviewed by Mann et al. [15]). It is well known that DCs in the gut are generally hyporesponsive [16] and have the ability to imprint homing properties on T cells. Although defining cell markers to identify intestinal DCs is controversial, there are different strategies to identify human DCs in the mucosa. The most common one is by negative lineage of CD3, CD14, CD16, CD34, and expression of $HLA\text{-}DR^+$. These cells are often mistaken as macrophages, and some authors prefer to differentiate both subsets by function and not by cell markers. Interestingly, $CD103^+$ DCs have tolerogenic properties and share some functional aspects with murine $CD103^+$ DCs. These "tolerogenic" DCs promote Treg differentiation [16] and produce RA [17] and indoleamine 2,3-dioxygenase (IDO) [18], molecules that can drive Tregs and are known to be involved in the induction of tolerance in the gut. The ability of $CD103^+$ DCs to produce IL-10 and the lack of IL-12, together with the low expression of CD40, TLR2, and TLR4, make these cells suitable to be defined as the main regulators of immune tolerance in the intestinal tract. However, research in this area is very difficult and a lot more needs to be elucidated regarding human DC subsets and tolerance in the gut.

3. Mechanisms to Induce Tolerance

The functional properties of DCs are dependent on their maturation status. Due to the lack of expression of costimulatory molecules and MHCII, tol-DCs are able to induce T cell anergy, preventing T cell activation. It has been described that DCs suboptimal antigen presentation, combined with the expression of IDO or FasL (CD95L) leads to inhibition of T cell proliferation and T cell deletion [19] (Figure 1). The induction of Treg and Type 1 regulatory T cells (Tr1) by DCs is another mechanism to induce peripheral tolerance [20, 21]. The mechanisms by which DCs regulate immune responses, tolerance, and lamina propria homeostasis against commensal flora have not been fully elucidated. The immunosuppressive cytokine IL-10 is a crucial mediator of tolerance in the gut and it has a nonredundant role in limiting inflammatory responses in the intestine. IL-10 can act on a variety of immune cells and its secretion is certainly involved in Tregs and Tr1 induction as well as regulating the local inflammatory immune response via antigen-presenting cells [22–24]. Indeed, IL-10 is very important in maintaining intestinal homeostasis as revealed by the spontaneous chronic

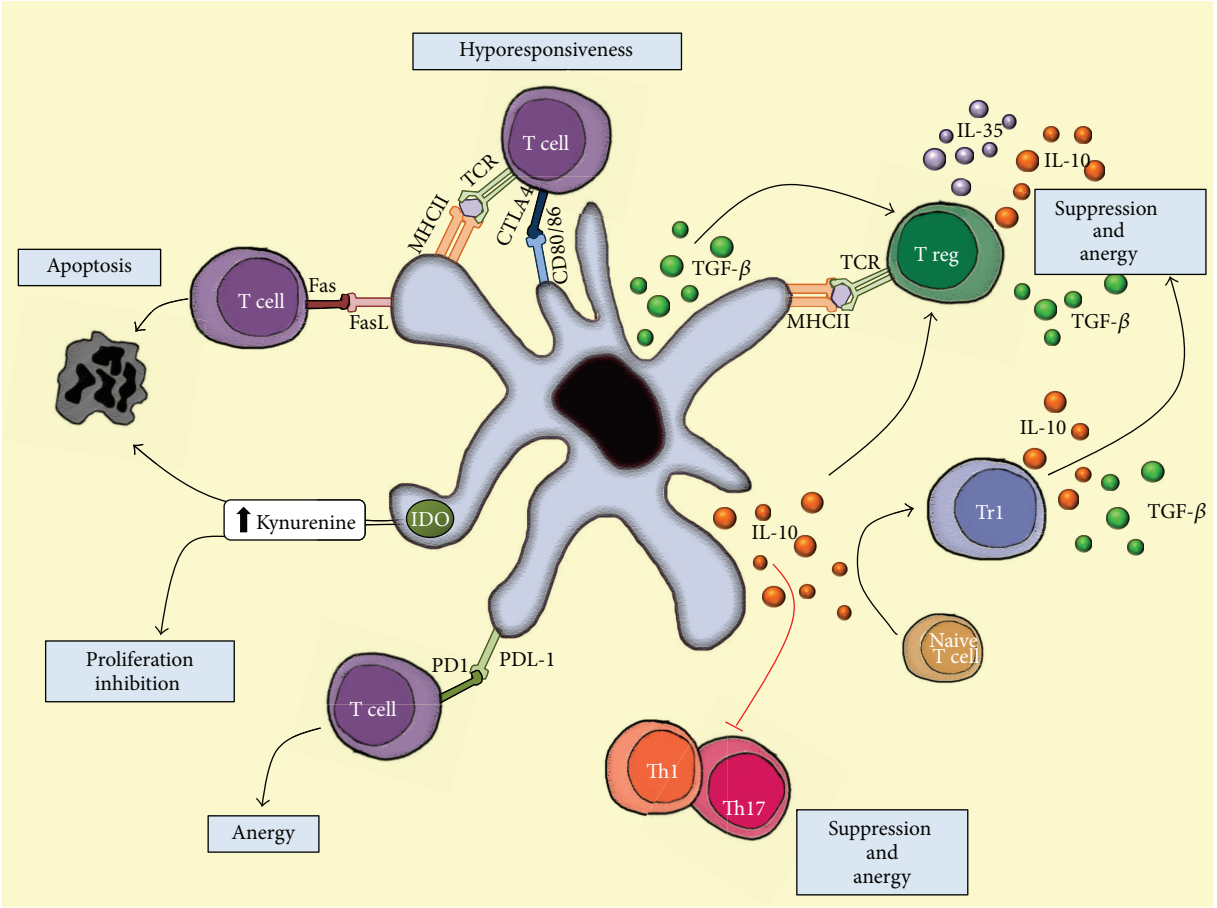

FIGURE 1: Summary of different mechanisms to induce tolerance by DCs; Treg and Tr1 cells generation, suppression of T effector cells, inhibition of proliferation, apoptosis induction, T cell anergy, and hyporesponsiveness.

inflammatory disease (similar to Crohn's disease) that IL-10 knockout mice develop. In addition, a severe form of Crohn's disease (with early-onsets of enterocolitis, involving hyperinflammatory immune responses in the intestine) in infants was associated with IL-10 receptor mutations in two unrelated consanguineous families [25]. IL-10 signaling directly suppresses Th17 and $Th17^{+}Th1^{+}$ cells in mice with established colitis [26]. All these features together make tol-DCs suitable to create an immunosuppressive environment that can potentially induce tolerance in the neighboring tissue.

4. Plasmacytoid Dendritic Cells and Tolerance

Plasmacytoid DCs (pDC) appear to play an important role in the regulation of tolerance induction [27], transporting self-antigens from peripheral tissues in the thymus contributing to the inactivation of autoreactive T cells and induction of Tregs [28, 29]. While present in tissues at very low numbers in the healthy steady-state, pDCs accumulate in lymphoid and nonlymphoid tissues under pathological or inflammatory conditions. In addition, the role of pDCs in controlling the intestinal homeostasis is largely unknown. Interestingly, liver and spleen pDCs express higher levels of NOD2 than conventional myeloid DCs (mDCs) and pDC are able to detect and respond to muramyl dipeptide (MDP). NOD2 ligation reduces IL-12, IL-6, and TNF-a production by pDC, in the presence or absence of either LPS or CpG stimulation [30]. Aberrant accumulation of pDCs in MLN and inflamed mucosa of IBD patients compared to controls has been shown [31]. Furthermore, highly purified pDCs from patients produced high levels of proinflammatory cytokines and showed an activated phenotype. However, IFN-α secretion induced by CpG-A was impaired in pDCs from IBD patients [31]. Another report showed that IBD patients lack circulating immature blood DCs (both DC subsets, myeloid, and plasmacytoid) during flares, which possibly migrate to the gut. An aberrant response to microbial surrogate stimuli suggests a disturbed interaction with commensals [32]. It has been recently shown that CCR9 expression in pDCs can home to the gut [33] and induce potent Treg responses that have a significant therapeutic effect in a model of intestinal Graft Versus Host Disease [34]. It is important to highlight that the clinical benefit of G-CSF therapy in Crohn's disease patients is thought to be related to its ability to induce IL-10-mediated regulatory functions, associated possibly with increased pDCs numbers in the inflamed gut [35]. However, a direct correlation between pDCs and the clinical benefit has not been established yet. Despite their reported role in the pathogenesis of certain autoimmune diseases, such as SLE or

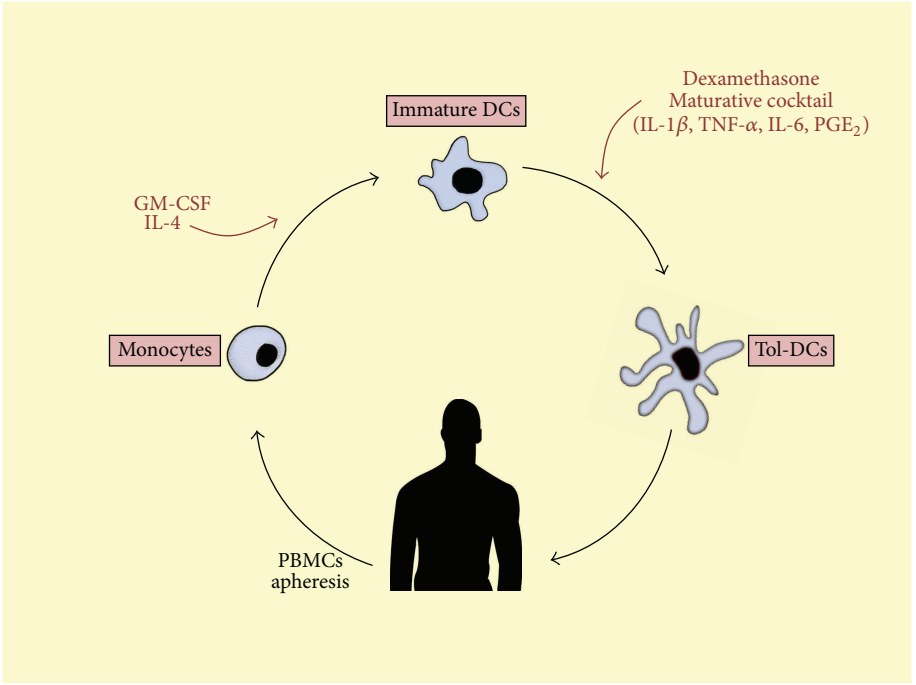

Figure 2: General scheme: dendritic cell therapy for Crohn's disease patients. Isolated monocytes are cultured and differentiated into DCs by adding IL-4 and GM-CSF to the media. At day 3, addition of dexamethasone induces the tolerogenic profile, and at day 6, addition of the maturation cytokine cocktail potentiates the tolerogenic properties.

psoriasis, understanding pDC function in the pathogenesis of human diseases has just begun.

5. Role of Tol-DCs in IBD Animal Models

Experimental data generated in murine models of colitis are highly promising especially relating to the ability of tol-DCs to prevent, reverse, or ameliorate established colitis [12, 36, 37]. In a model of TNBS-induced colitis, which closely parallels the immune activation in Crohn's disease, injection of tol-DCs treated with Vasoactive Intestinal Peptide (VIP) [38] significantly ameliorated the clinical and histopathology severity of colitis in mice. An important aspect of this study was the route of administration of the DCs; the authors clearly show that by intraperitoneal administration DCs gain access to mesenteric lymph nodes, where the most important antigen presentation and activation of Th1/Th17 cells takes place [39]. In addition, different types of tol-DCs generated with a combination of dexamethasone plus vitamin D3 [12] or loaded with enterobacterial extract [36] were able to prevent the colitis induction. Several other animal models have revealed the therapeutic role of tol-DCs in preventing and ameliorating IBD in an antigen-specific way [37]. However, the current challenge is to bring this tol-DC therapy to the clinic for human patients. Several issues must be overcome such as the difference between IBD-induced animal models (reviewed by Neurath [40]) and the human disease, or the functional differences between mouse and human DCs. In summary, those promising results in rodents await to be translated into the human application.

6. Therapeutic Application of Tol-DCs in Crohn's Disease Patients

We have developed a protocol to produce tol-DCs under clinical grade conditions for the treatment of Crohn's disease patients (Figure 2) by conditioning monocyte-derived DC with dexamethasone at day 3, together with 24 hours maturation with a cytokine cocktail (IL-1β, IL-6, and TNF-α) plus PGE_2 [41]. Compared to mature and immature DCs, our tol-DCs produced higher levels of IL-10, even in response to gram-negative bacteria or synthetic LPS, with low or undetectable levels of IL-12p70, IL-23, or TNF-α. In addition, tol-DCs phenotype was consistently semimature with intermediate expression of costimulatory receptors (CD80 and CD86), low levels of CD83 and MHC class II, and the ability to inhibit T cell responses. It has been shown that DC activation with LPS or the clinical grade TLR4 ligand MPLA enhances tol-DCs migratory properties and antigen presentation capabilities [42]. Interestingly, even though the fact that isolated monocytes from Crohn's disease patients are in an enhanced proinflammatory environment [32], we showed that these cells from Crohn's disease patients can be educated towards tolerogenic phenotype. These results are in line with studies in other immune-based diseases like rheumatoid arthritis or multiple sclerosis [43, 44]. This is a key aspect for considering this DC-based treatment as a therapeutic option in IBD, because it might have occurred that genetic variants conferring susceptibility for Crohn's disease or the proinflammatory environment might alter the biology of DCs.

7. Lack of Crohn's Disease Associated Antigen

DC-based therapies are envisaged to inhibit antigen-specific T cell responses, and the appropriated antigen selection to load DCs is under intensive research. Although humoral response against antigens derived from microbiota has been described in Crohn's disease patients, for example, elevations in anti-Saccharomyces cerevisiae antibodies (ASCA) in 49–60% of cases [45], no T lymphocyte Crohn's disease-specific antigen has been properly identified. Although the disease is associated with a high inflammatory component, mainly corresponding to Th1 and Th17 T cells [38, 39], the antigenic specificity of these cells remains to be investigated. Interestingly, commensal-specific T cell responses are detected during mouse model of intestinal inflammation with *Toxoplasma gondii* infection [46, 47]. It is tempting to speculate that commensal specific T cells may represent an important component of the IBD, although much remains to be understood about this issue. In animal models, Yamanishi et al. [37] identified a specific protein, carbonic anhydrase I (CA I), specifically involved in the IBD pathogenesis. Interestingly, the authors demonstrate the role of CA I loaded tol-DCs in preventing the induction of colitis via Tregs. Pedersen et al. administered DCs pulsed with enterobacterial extract to suppress development of colitis [36]. However, other authors have demonstrated tolerance induction in colitis model without antigens [12, 38]. This mechanism would involve the generation of DCs secreting regulatory cytokines (TGF-β and IL-10) and expressing inhibitory receptors that might overcome the necessity of a known antigen. This "transtolerance" may result in the generation of a specific regulatory response helping to restore the mucosal homeostasis.

8. Summary

DCs are powerful therapeutic tools to modify the immune response and restore the immune tolerance in Crohn's disease patients and other autoimmune diseases. An alternative to manipulate the different subsets of intestinal DC function is the *in vitro* generation of tol-DCs. Methods to obtain these cells in sufficient amounts have been developed. Tol-DCs may represent a new therapeutic strategy for Crohn's disease, where the alterations of the finely tuned balance between the immune system and the microflora result in disease. Several reports have indicated the therapeutic effect of tol-DCs in inhibiting IBD induction in animal models. These results highlight the importance of DCs in the intestinal homeostasis control and open new avenues for an innovative therapeutic indication for human patients.

Conflict of Interests

The authors declare no existing financial conflict of interests.

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Research Article

Persistent Suppression of Type 1 Diabetes by a Multicomponent Vaccine Containing a Cholera Toxin B Subunit-Autoantigen Fusion Protein and Complete Freund's Adjuvant

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Data presented here demonstrate multifunctional vaccination strategies that harness vaccinia virus mediated delivery of a gene encoding an immunoenhanced diabetes autoantigen in combination with complete Freund's adjuvant (CFA) that can maintain safe and durable immunologic homeostasis in NOD mice. Systemic coinoculation of prediabetic mice with recombinant vaccinia virus rVV-CTB::GAD and undiluted or 10-fold diluted CFA demonstrated a significant decrease in hyperglycemia and pancreatic islet inflammation in comparison with control animals during 17–61 and 17–105 weeks of age, respectively. Synergy in these beneficial effects was observed during 43–61 and 61–105 wks of age, respectively. Inflammatory cytokine and chemokine levels in GAD-stimulated splenocytes isolated from vaccinated mice were generally lower than those detected in unvaccinated mice. The overall health and humoral immune responses of the vaccinated animals remained normal throughout the duration of the experiments.

1. Introduction

Type 1 diabetes mellitus (T1D) is a chronic metabolic disease that is based on autoimmunity and is most frequently initiated in childhood. Initial symptoms include autoreactive lymphocyte mediated progressive destruction of the insulin-producing beta islet cells of the pancreas triggered by the innate and ultimately the adaptive arm of the body's immune system. This early perturbation of immunological homeostasis results in a progressive loss of islet β-cell function, leading to an overall insulin deficiency and resulting in elevated blood sugar levels (hyperglycemia), increased cellular oxidative stress leading to chronic pancreatic islet inflammation, and an associated risk for development of secondary neural and circulatory health problems, resulting in amputation of the extremities, blindness, and increased probability of heart attack and stroke [1]. Type 1 diabetes incidence is steadily increasing in the western world [2]. In the United States, approximately 3 million Americans are afflicted with all forms of diabetes, of which from 15 to 20% currently suffer from T1D. Showing the extensive nature of this disease, more than 13,000 children are diagnosed with T1D in the U.S. annually. Hyperglycemia, the major manifestation of clinical diabetes, represents the final outcome of immunological processes that have progressed over a number of months in mice and years in humans. Treatments for disease prevention which focus on arresting or reversing hyperglycemia are inadequate, as islet β-cell destruction is completely asymptomatic until more than half of the approximately 1×10^6 islets in the human pancreas have been irreversibly inactivated or destroyed. Familial inheritance studies show that genetic factors play a significant role in T1D development, and at least 15 genetic loci have been linked to T1D susceptibility in the nonobese diabetic (NOD) mouse model [3]. However, since type 1 diabetes occurs in only approximately 50% of monozygotic

Table 1: NOD mouse treatment groups for rVV-mediated suppression of hyperglycemia.

Groups	Treatment (i.p. and s.c. inoculation)	PFU (rVV)	Age at injection
1 ($n = 10$)	PBS (naive)	0	5 and 7 wks
2 ($n = 10$)	CFA (1 : 0)	0	5 and 7 wks
3 ($n = 10$)	CFA (1 : 10)	0	5 and 7 wks
4 ($n = 10$)	CFA (1 : 100)	0	5 and 7 wks
5 ($n = 10$)	rVV-CTB::GAD + CFA (1 : 0)	$2 \times (5 \times 10^7)$	5 and 7 wks
6 ($n = 10$)	rVV-CTB::GAD + CFA (1 : 10)	$2 \times (5 \times 10^7)$	5 and 7 wks
7 ($n = 10$)	rVV-CTB::GAD + CFA (1 : 100)	$2 \times (5 \times 10^7)$	5 and 7 wks
8 ($n = 10$)	rVV-CTB::GAD	$2 \times (5 \times 10^7)$	5 and 7 wks

twins, genetic risk factors are insufficient to account for disease occurrence [4]. Environmental factors, including virus infection and dietary components, are thought to contribute to diabetes onset [5, 6]. Following $CD4^+$ autoreactive T helper (Th) cell infiltration of pancreatic islets in NOD mice, autoreactive effector Th1 lymphocytes were shown to secrete inflammatory cytokines IFN-γ and IL-2. These cytokines stimulate macrophage and CTL secretion of oxidative compounds and inflammatory cytokines that induce chronic pancreatic inflammation (insulitis) and were shown to contribute to the apoptosis of islet insulin-producing β-cells [3]. A variety of immune cells including dendritic cells, macrophages, natural killer (NK) cells, and B cells have also been shown to participate in diabetes pathogenesis [5]. B cells influence the developing autoimmune T-cell response mainly in the initial stages of T1D development [7]. At present, there is no established clinical approach that can effectively suppress long-term T1D. However, a sufficient number of β-cells may exist at the time of diagnosis of T1D, and diabetes could be reversed once the autoimmune response is rapidly suppressed. Recent studies show that the development of a vaccine that prevents autoreactivity or reestablishes immune regulation once autoreactivity occurs may provide a promising therapy for T1D treatment. A list of the major autoantigens in T1D includes insulin, glutamic acid decarboxylase (GAD), insulinoma antigen (IA-2), and several other islet beta cell proteins [8]. In general, two strategies of molecular vaccination have been developed: viral vector-based and recombinant plasmids, both carrying genes for autoantigens, and/or immunomodulatory proteins. In our earlier studies, partial diabetes suppression was observed following vaccinia virus (rVV-CTB::GAD) mediated mucosal or intraperitoneal inoculation of NOD mice with CTB::GAD fusion protein [9]. Recently we demonstrated that a combination of rVV-CTB::GAD with the rVV-IL10 virus expressing the anti-inflammatory cytokine IL-10 was effective in preventing diabetes onset in NOD mice [10]. Complete Freund's adjuvant (CFA) containing heat-killed mycobacteria (*M. tuberculosis*) demonstrated beneficial effects in the prevention of diabetes onset in NOD mice [11]. While the underlying mechanism remains unknown, CFA may act in part by enhancing the ability of NOD mouse antigen presenting cells activation of NOD $CD4^+$ $CD25^+$ regulatory cells responsible for the control of autoreactive T-cells and prevention of disease in NOD mice [12]. However, the level of immune system stimulation from normally inoculated doses of CFA may cause unacceptable side effects. Therefore, here we investigate whether virus-delivered CTB::GAD treatment in combination with reduced CFA dosages can provide effective, durable, and safe prevention in prediabetic NOD mice.

2. Materials and Methods

2.1. Multicomponent Vaccine Construction. The vaccinia virus construct rVV-CTB::GAD used in this study contains a cDNA fragment encoding the diabetes pancreatic islet autoantigen GAD55, made up of a truncated form of human GAD65 minus the N-terminal membrane binding region (aa 89–585) linked to the C-terminus of the cholera toxin B subunit gene (CTB), as previously described in [9]. Complete Freund's adjuvant (CFA) was purchased from Sigma-Aldrich Co. (St. Louis, Mo). Each mL of adjuvant contained 1 mg of *Mycobacterium tuberculosis* (H37Ra, ATCC 25177), heat killed and dried, 0.85 mL paraffin oil, and 0.15 mL mannide monooleate. CFA was diluted 1 : 10 and 1 : 100 in phosphate-buffered saline (PBS).

2.2. Detection of Hyperglycemia in Immunized NOD Mice. Four-week-old female NOD LtJ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal care facility of the Central Veterinary Institute, Budapest, Hungary. The protocol for mouse rVV inoculation was approved by the Animal Research and Care Committees of Loma Linda University School of Medicine (Loma Linda, CA) and the Central Veterinary Institute in Hungary. Prior to measurement of hyperglycemia, a total of eight experimental groups of 5-week-old NOD mice ($n = 10$) were subjected to subcutaneous (s.c.) injection with 0.1 mL of undiluted, 1 : 10 and 1 : 100 diluted CFA, or/and intraperitoneal (i.p.) inoculation with 0.3 mL rVV-CTB::GAD (5×10^7 PFU/mL). Two weeks after the first injection, the vaccine inoculations were repeated. One mock-infected experimental group ($n = 10$) was inoculated with PBS only.

The mice were fed complete mouse chow and water *ad libitum*. To detect the onset of hyperglycemia, beginning at 13 wks of age, the mice in each experimental group (Table 1) were bled from the tail vein biweekly and blood sugar levels were quantified. Diabetes was confirmed when blood glucose levels exceeded 14 mmol/L for two consecutive weeks. Blood

glucose levels were confirmed with Keto-Diastix urinary glucose test strips (Bayer AG, Leverkusen, Germany). Experiment was completed at 105 wks of age. After that, in selected experimental groups, indications related to aging (tumors, liver degenaration, and heart problems) were observed. The incidence of hyperglycemia was considered to be insignificant between experimental groups when the calculated Z value was between −1.96 and +1.96. The Mann-Whitney U test was also applied to compare hyperglycemia incidence and blood glucose levels among groups. Statistical significance was determined at $P < 0.05$. A synergistic effect in the combined two-component treatment (CTB::GAD + CFA) was considered when each component alone (rVV-CTB::GAD or CFA) did not show a statistically significant difference compared to naive mice, while the combined treatment (rVV-CTB::GAD + CFA) did result in a statistically significant difference in comparison with naive mice. Additive effect is observed when both components alone, as well as combined treatments result in statistically significant differences compared to naive mice. The difference in effects conferred by naive and combined two-component-treated mice is larger than that conferred by each component alone.

2.3. Histopathological Analysis and Computer-Assisted Morphometry Measurement of Pancreatic Islets. In this study pancreatic islets of hyperglycemic and euglycemic mice were analyzed separately. Mice that developed blood glucose concentrations of 33 mmol/L (hyperglycemic) were sacrificed and the extent of lymphocyte islet infiltration was evaluated, as previously described in [10]. Mice that did not develop hyperglycemia over the course of the experiment (euglycemic mice) were sacrificed for histopathological analyses at 119 wks of age. The degree of insulitis was measured in each mouse based on the extent of lymphocyte infiltration of the islets. The percentage of the infiltrated area was measured using AxioVision 4 microscope software (Carl Zeiss Inc., Jena, Germany). Insulitis scores were based on a 5-level semiquantitative scale ranging from 0 to 4, where an insulitis score of 0 was considered to be normal regarding islet morphology, with no indication of autoreactive lymphocyte infiltration. Insulitis scores of 1-2 indicated progressively increasing levels of peri-islet insulitis and scores of 3-4 indicated progressive levels of intraislet insulitis, with a score of 4 equivalent to complete invasion of the islet by autoreactive lymphocytes [9].

2.4. Analyses of Secreted Cytokines/Chemokines and T-Cell Subsets of Splenocytes. The mice were sacrificed by CO_2 asphyxiation and the spleens immediately excised. The splenocytes were isolated, as described in [10]. Briefly, the spleens were frozen in 90% FBS and in 10% DMSO solution and were stored at −196°C until examination. Prior to testing, frozen splenocyte samples were thawed rapidly by warming in a 37°C water bath, diluted with 25 mL of RPMI 1640 containing 10% FCS, collected by centrifugation at 250 g for 10 min, and suspended in 10 mL RPMI 1640 with 10% FCS. The splenocyte samples were then transferred into 25 cm^2 tissue culture flask with vented cap (Sarstedt, Inc., Newton, NC) and incubated at 37°C overnight. On the next day, 1 × 10^7 cells/mL splenocytes of the mice group naive, CFA (1 : 0), rVV-CTB::GAD + CFA (1 : 0), and rVV-CTB::GAD + CFA (1 : 10), were stimulated with GAD65 peptide (30 μg/mL) comprised of amino acids 530 to 543 of the protein (AnaSpec, Inc., Fremont, CA), or without the GAD peptide (data not shown) as a control. Stimulation was performed in 15 mL polypropylene tubes held at an angle of 5 degrees for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Following incubation, the splenocyte preparations were centrifuged at 350 g for 10 min at room temperature to sediment the cells. The supernatant culture medium was collected and stored at −80°C until examination for secreted cytokine content. The splenocytes were used immediately for the flow-cytometric analysis of the T-cell subsets.

For examination of $CD3^+$, $CD4^+$, and $CD8^+$ surface markers, splenocytes were stimulated (or not in the controls, data not shown) with the GAD65. Cells then were suspended in 2 mL of PBS and centrifuged at 300 g for 5 min at room temperature. The sedimented cells were suspended again in an appropriate volume of PBS (1 × 10^7 cells/mL). Splenocytes were stained with a three-color reagent—Mouse T Lymphocyte Subset Antibody Cocktail, with Isotype Control (BD Pharmingen, San Jose, CA), designed to identify major subsets of T lymphocytes by direct immunofluorescent staining with flow cytometric analysis. This cocktail consisted of a mixture of PE-Cy7 hamster anti-mouse CD3e, PE rat anti-mouse CD4, and FITC rat anti-mouse CD8a antibodies. An equivalent concentration of fluorochrome- and isotype-matched negative-control immunoglobulins was used as the Mouse T Lymphocyte Subset Isotype Control. Subsequently, the samples were vortexed and incubated for 30 min at room temperature in the dark, and then the cells were centrifuged at 300 g for 5 min at room temperature. After removing the supernatant, the sedimented cells were suspended in 2 mL of BD CellWASH solution (BD Biosciences, San Jose, CA) followed by centrifugation at 300 g for 5 minutes at room temperature. The supernatants were removed and cells were fixed by 0.5 mL of CellFIX solution and flowcytometry was performed using a BD FACSCalibur flow cytometer (BD Biosciences). The one-way Anova was used to evaluate the statistical significance of differences in the percentage of positive cells. Experimental values were considered significant at $P < 0.05$.

The supernatants of the splenocytes stimulated with GAD65 peptide of mice inoculated with PBS, CFA (1 : 0), rVV-CTB::GAD + CFA (1 : 0), and rVV-CTB::GAD + CFA (1 : 10), were used for the analyses of cytokine/chemokine secretion. The relative levels of selected mouse cytokines and chemokines were determined using Proteome Profiler Array, Mouse Cytokine Array Panel A kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instruction. Briefly, the membranes were transferred into a 4-well multidish and were blocked in Array Buffer 6 by incubation for one hour on a rocking platform. One mL of the pooled supernatant samples from each group was transferred to 0.5 mL of Array Buffer 4 in separate tubes. Reconstituted Mouse Cytokine Array Panel A Detection Antibody Cocktail (15 μL) was added to each prepared sample. The samples were mixed and incubated at room temperature for one

hour. After removing the Array Buffer 6 from the 4-well multidish, the sample/antibody mixtures were added to the membrane and were incubated overnight at 2–8°C on a rocking platform shaker. Following the incubation, each membrane was washed two times with a 1x wash buffer for 10 minutes on a rocking platform shaker. Two mL of diluted in Array Buffer 6 Streptavidin-HRP were pipetted into each well of the 4-well multidish, and then the membranes were returned to the Multi-dish and were incubated for 30 minutes at room temperature on a rocking platform shaker. Following the incubation, the membranes were washed as described above, and then 1 mL of the prepared Chemi Reagent Mix was added evenly onto each membrane. The membranes were placed in an autoradiography film cassette, and were exposed to X-ray film for 1, 5, and 10 minutes. Pixel densities on developed X-ray film were collected and analyzed using a transmission-mode scanner and image analysis AxioVision 4 software.

2.5. Detection of IL-10 and IL-12 in NOD Mouse Serum. The BD Mouse IL-10 Flex Set and the BD CBA Mouse IL-12p70 Flex Set Bead Based Immunoassay were used to measure IL-10 and IL-12 levels in serum samples, respectively. The sets were used in conjunction with a BD CBA Mouse/Rat Soluble Protein Master Buffer kit, a BD FACSCalibur flow cytometer, and the FCAP array software according to the manufacturer's instructions (all from BD Biosciences). In brief, 50 μL of the mixed capture beads was transferred into each assay tube. Serum samples (50 μL) were diluted 1 : 4 in assay diluent and transferred to the assay tubes. The tubes were incubated in the dark for 2 h at room temperature. Following the incubation, a mixed PE detection reagent was added to each assay tube (50 μL/test) and incubated in the dark for 1 h at room temperature. The samples were washed with 1.0 mL of wash buffer followed by centrifugation at 200 g for 5 minutes. The supernatant was removed and 300 μL of Wash Buffer was added to the assay tubes and was briefly vortexed before analyzing samples by flow cytometry. In addition, a serial dilution of the BD CBA Mouse or Rat Soluble Protein Flex Set Standard was used to establish a standard curve for accurate determination of the secreted cytokine levels in each sample. The Student's t-test was used to evaluate the statistical significance of differences in the serum cytokine levels. Experimental values were considered significant at $P < 0.05$.

2.6. VV-Specific Antibody Induction. To optimize the enzyme-linked immunosorbent assay (ELISA), a VV-specific monoclonal antibody (3B10/G9/B7) against VV A33R gene product (unpublished) was used as a positive control (not shown). Serum of naive Balb/c mice was used as a negative control. The working dilution of the VV-antigen was determined by titration in carbonate buffer (pH 9.6), NaOH (pH 13), 0.1 M Glycin (pH 2.7), and PBS (pH 7.2). To measure virus-specific IgG by ELISA, the antigen was diluted with NaOH (pH 13), and then 100 μL aliquots were measured into the wells of the ELISA plate (Analyzer Ltd., Budapest, Hungary). After incubation at +4°C overnight, the plates were washed five times with PBS washing-diluting buffer containing 0.05% Tween 20 (Sigma Aldrich Co., St. Louis, MO), and then 100 μL volumes of the sera diluted 1 : 5 with PBS-Tween 20 buffer were measured in the wells. The plates were incubated at +37°C for 60 min and then washed five times with PBS-Tween 20 buffer. Subsequently, 200 μL rabbit anti-mouse IgG (H+L) horseradish peroxidase (HRP) conjugate (Jackson Immuno Research Labs Inc., West Grove, PA) diluted 1 : 2000 in PBS-Tween 20 buffer was measured in the wells. After a 60 min incubation at +37°C, the plates were washed as described above, and then the enzyme activity was visualized by the addition of 100 μL of tetramethylbenzidine (TMB) (Diavet Ltd., Budapest, Hungary). After a 20 min incubation at room temperature, the colored reaction was stopped by addition of 50 μL of 2 N H_2SO_4 solution per well. Optical density (OD) of samples was measured at 450 nm in a Multiscan Ms reader spectrophotometer (Labsystems Oy, Helsinki, Finland). In addition, each assay plate also contained a positive and negative control as described above. The Mann-Whitney U test was used to evaluate the statistical significance of differences in the optical density values. Experimental values were considered significant at $P < 0.05$.

3. Results

3.1. Suppression of Hyperglycemia in NOD Mice. Undiluted (1 : 0) and 10- and 100-fold diluted CFA adjuvant was used in NOD mouse inoculation studies in combination with the recombinant virus rVV-CTB::GAD to assess the efficacy of the multifunctional vaccine in autoantigen-dependent enhancement of immune suppression of T1D symptoms of hyperglycemia and insulitis. Following inoculation of NOD mice with the CFA alone, the frequency of hyperglycemia in the mouse experimental groups increased at statistically significant rates depending on the nature of the treatment. The incidence of diabetes diagnosed in the control PBS group and in the CFA 1 : 100 experimental group rapidly increased from 13 wks until 31 and 33 weeks of age, respectively, reaching 90% and 80% in diabetes incidence, respectively (Figure 1(a)). At termination of the experiment, 9 of 10 PBS- or CFA 1 : 100-inoculated mice (90%) developed terminal diabetes at 31 and 61 wks, of age, respectively.

These experimental data indicate that the lowest dose of CFA (1 : 100) alone had no measurable effect on suppression hyperglycemia in the vaccinated mice. The 1 : 10 dilution of CFA alone generated an intermediate level of diabetes protection during 19 and 31–37 wks of age as statistically confirmed by "test for equality of two proportions," in comparison with the naive control group (PBS) (Figure 1(a) and Table 2). Mice in this group gradually attained 80% hyperglycemia, which stimulated morbidity by 47 wks and 100% hyperglycemia, which stimulated morbidity by 93 wks of age. Inoculation with undiluted (1 : 0) CFA produced partial protection during 17–59 wks of age ($P < 0.01$), in comparison with the naive group (PBS), with a gradual increase in hyperglycemia to 40% by 37 wks of age and reaching a final level of 60% morbidity by 77 wks of age (Figure 1(a) and Table 2). Thus, both undiluted

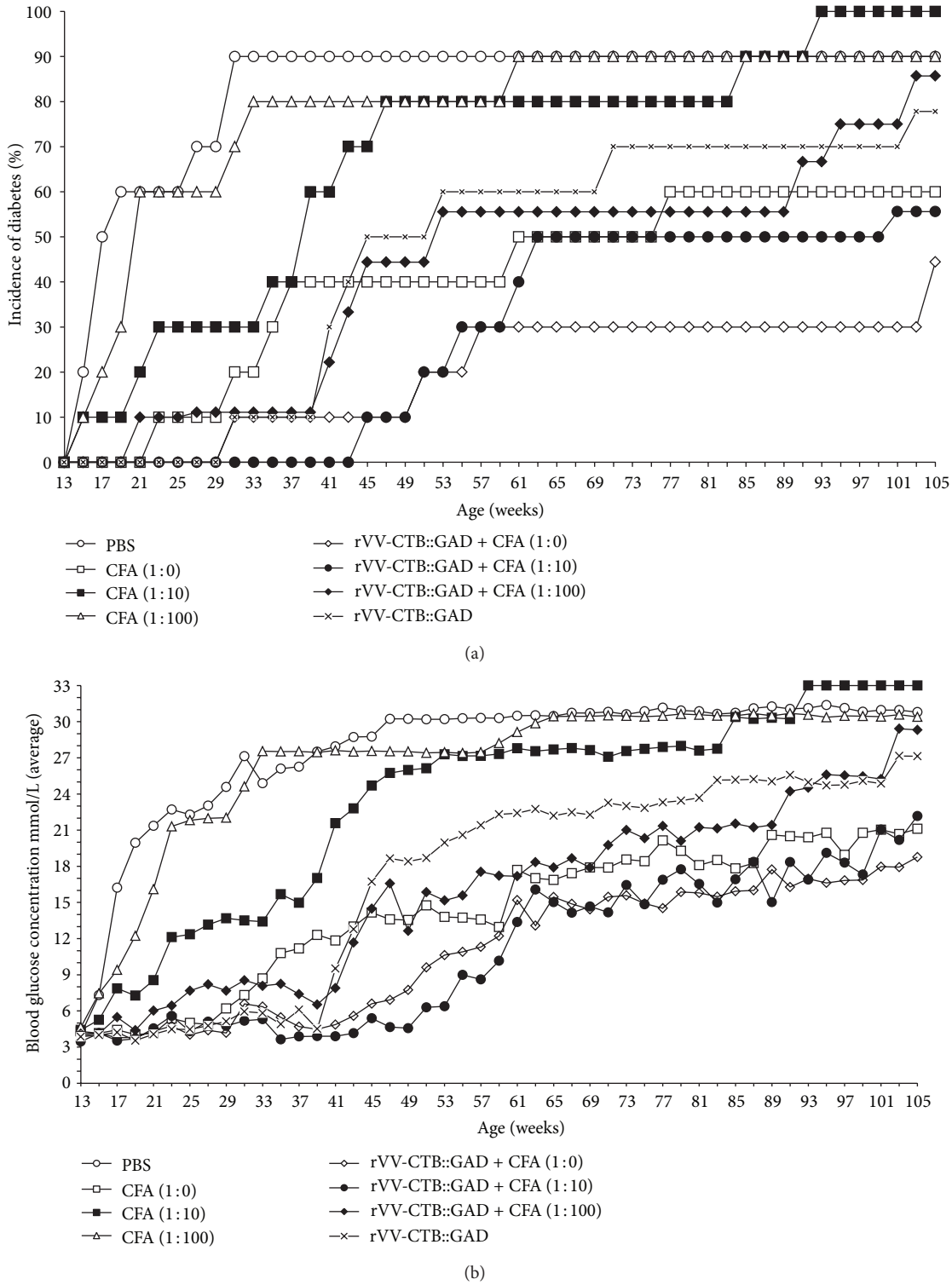


Figure 1: Incidence of diabetes in nonobese diabetic (NOD) mice. (a) Comparison of immunological suppression protocols on diabetes onset in NOD mice as determined by increased incidence of hyperglycemia. Treatment with rVV-CTB::GAD alone and coinoculation with CFA demonstrated statistically significant differences ($P < 0.01$–0.001) compared to the phosphate-buffered saline (PBS) (mock-infected) control group. Differences between CFA (1:10), CFA (1:100), and PBS treatment groups are statistically insignificant (Mann-Whitney U test). (b) Blood glucose levels in all experimental animal groups were monitored biweekly. Each data point represents the average blood glucose determination for all mice in that group until 105 weeks of age. Starting from 107 weeks of age several mice have died from unknown and unrelated to diabetes reasons; the figure does not show data points between 107 and 119 weeks. By the end of the experiment, highest blood glucose levels were detected in naive mice inoculated with PBS, CFA (1:10), and CFA (1:100), reaching average levels of 31, 33, and 30 mmol/L, respectively. Lowest levels of blood glucose maintenance were found in NOD mice inoculated with rVV-CTB::GAD + CFA (1:0) (18.8 mmol/L), CFA (1:0) (21.1 mmol/L), and rVV-CTB::GAD + CFA (1:10) (22.2 mmol/L). Differences in the average blood glucose levels in all groups were statistically significant ($P < 0.05$–0.001) compared to naive controls, except group of mice inoculated with CFA (1:100) (Mann-Whitney U test).

Table 2: Analysis of hyperglycemia development.

	Treatment	PBS (naive)	CFA (1 : 0)	CFA (1 : 10)	CFA (1 : 100)	rVV-CTB::GAD + CFA (1 : 0)	rVV-CTB::GAD + CFA (1 : 10)	rVV-CTB::GAD + CFA (1 : 100)	rVV-CTB::GAD
Groups		1	2	3	4	5	6	7	8
					P values/weeks*				
					Incidence of diabetes				
1	PBS (naive)		<**0.01**/17–59 wks	≥ 0.05/19 wks and 31–37 wks	≥0.05/none	<**0.01**/ 17–105 wks	<**0.001**/ 17–61 wks	<**0.01**/ 17–51 wks	<**0.01**/ 17–43 wks
2	CFA (1 : 0)	<**0.001**		≥0.05/ none	<**0.01**/ 21–35 wks	≥0.05/ none	≥0.05/ 37–43 wks	≥0.05/ none	≥0.05/ none
3	CFA (1 : 10)	<**0.05**	<**0.001**		≥0.05/ 33 wks	<**0.01**/ 39–105 wks	<**0.01**/ 35–59 wks and 93–99 wks	≥0.05/ 39 wks	≥0.05/ 39 wks
4	CFA (1 : 100)	≥0.05	<**0.001**	≥0.05		<**0.01**/ 21–105 wks	<**0.01**/ 21–61 wks	<**0.05**/ 21–43 wks	<**0.01**/ 21–43 wks
5	rVV-CTB::GAD + CFA (1 : 0)	<**0.001**	<**0.05**	<**0.001**	<**0.001**		≥0.05/ none	≥0.05/ 103 wks	≥0.05/ 103 wks
6	rVV-CTB::GAD + CFA (1 : 10)	<**0.001**	<**0.05**	<**0.001**	<**0.001**	≥0.05		≥0.05/ 43 wks	≥0.05/ 43 wks
7	rVV-CTB::GAD + CFA (1 : 100)	<**0.001**	<**0.05**	<**0.001**	<**0.001**	<**0.001**	<**0.001**		≥0.05/ none
8	rVV-CTB::GAD	<**0.001**	<**0.001**	<**0.001**	<**0.001**	<**0.001**	<**0.001**	≥0.05	
					Blood glucose				

* *P* value for each pair of groups was determined using two-tailed Mann-Whitney *U* value. Test for equality of two proportions was used to determine those weeks when statistically significant differences (*Z* values between −1.96 and +1.96) were detected.

1 : 0- and 1 : 10-diluted CFAs alone exert a protective effect on NOD mouse development of diabetes.

Inoculation with rVV-CTB::GAD alone provided a measurable level of protection similar to that of undiluted CFA (Figure 1(a)). The protective effects of the vaccine in this experimental group were measurable until 39 wks of age. Following 39 wks, the incidence of diabetes gradually increased to 70% at 71 wks of age. As expected, addition of CFA at a dilution of 1 : 100 did not provide improvement in CTB::GAD-mediated immune suppression of hyperglycemia. However, the combination of rVV-CTB::GAD with CFA diluted 1 : 10 substantially delayed hyperglycemia onset until 43 wks of age, after which animal morbidity gradually increased to 50% at 63 wks of age. In comparison with the control group (PBS), significant differences were measurable from 17 through 61 wks of age (Table 2). The lowest levels of hyperglycemia onset were observed following inoculation of the mice with CTB::GAD + undiluted CFA (1 : 0). Complete vaccine protection of the experimental animals was detected from 13 through 29 wks of age. However, from 29 to 57 wks of age, the level of hyperglycemia gradually increased to 30%, a level which was maintained until 103 wks of age. Statistically significant differences between the vaccinated experimental animal groups and the PBS experimental group were observed over the entire duration of the experiment (105 wks, see Table 2). Synergy in beneficial effects following treatment with the two vaccine components was observed following inoculation with rVV-CTB::GAD + CFA (1 : 10)—from 43 through 61 wks of age. Following vaccination with rVV-CTB::GAD + CFA (1 : 0) synergistic immunosuppressive effects were observed from 61 through 105 wks of age. The additive immunological suppression was observed for the rVV-CTB::GAD + CFA (1 : 10) experimental group between 17 and 43 wks of age, while for the rVV-CTB::GAD + CFA (1 : 0) treatment group the effect was observed from 17 through 59 weeks of age.

Data on blood glucose levels of various treatment groups of NOD mice are presented in Figure 1(b). Elevated glucose levels (averaging 7.32 mmol/L) were detected in the PBS control and CFA diluted 1 : 100 alone groups of mice as early as 15 wks of age. In mice inoculated with CFA 1 : 10 alone, similar blood sugar levels (7.87 mmol/L) were detected two weeks later, at 17 wks of age. However, in the following weeks, mice in this group developed hyperglycemia at a retarded level in comparison with CFA (1 : 100). The experimental group of mice inoculated with undiluted CFA alone showed increased blood sugar levels starting at 23 wks of age. However, in the experimental animal group coinoculated with CTB::GAD +

CFA (1 : 0 or 1 : 10 dilution), elevated blood sugar levels were not detected until 31 and 43 wks of age, respectively (see statistical analyses in Table 2). Among mice inoculated with CTB::GAD, elevated blood sugar levels were detected as early as 29 wks of age.

Analyses of individual animals in all treatment and control groups are shown in Figure 2. In the control group (Figure 2(a)), only one mouse (no. 2) remained diabetes-free until the end of the experiment, showing an increase in blood sugar levels only after 71 weeks of age. However, clinical diabetes was not confirmed by urinary glucose testing (Figure 2(a)). The remaining nine mice developed diabetes indicating a relatively low level of genetic variability in experimental NOD mice. Six of nine mice became diseased by 19 wks, while the remaining three mice became hyperglycemic at 27 (no. 6) and 31 (no. 7-8) wks. Among mice inoculated with CFA 1 : 10 or CFA 1 : 100 alone, the first sick animals were detected as early as 15 wks of age. Otherwise, the pattern observed in the CFA (1 : 100) group (Figure 2(d)) was similar to that of the PBS group with the only difference being that the last mouse in this group developed diabetes with a significant delay, at 61 wks of age. From these data we can confirm the experimental results presented above indicating that the highly diluted CFA (1 : 100) alone has no detectable therapeutic effect. In contrast, the low dose of CFA (1 : 10) and undiluted CFA (1 : 0) were shown to provide beneficial effects in the prevention of diabetes onset, especially in the case of undiluted CFA (Figure 2(b)). By 21 weeks of age, none of the total 10 mice had developed diabetes. Most of the intermediate-dose CFA (1 : 10) treated mice (8 out of 10) gradually developed diabetes from 15 to 47 weeks of age (Figure 2(c)). Similarly, a moderately beneficial effect was observed in rVV-CTB::GAD and rVV-CTB::GAD + CFA (1 : 100) groups (Figures 2(g) and 2(h)). Interestingly, all cases of morbidity within the rVV-CTB::GAD + CFA (1 : 0) group, except for no. 5 (105 wks), fall between 31 and 57 weeks of age. Among mice treated with rVV-CTB::GAD + CFA (1 : 10), five animals developed diabetes at a later time, between 45 and 63 weeks of age (Figures 2(e) and 2(f)) as expected. All the mice analyzed that developed high blood glucose levels also generated a high intraislet insulitis of score = 4 (data not shown). Of 13 euglycemic mice analyzed, 4 mice (30.8%) developed peri-islet insulitis with scores of 1 and 2, and 9 mice (69.2%) developed intraislet insulitis with scores of 3 and 4 (Figure 3).

For analyses of splenocytes, during the experiments 4-5 mice from four groups, control (PBS), and three treatment groups, were euthanized. The synthesis of cytokines and chemokines in GAD65-activated splenocytes of each mouse was analyzed using Mouse Cytokine Array Panel A kit of the Proteome Profiler Array (R&D Systems, Inc., Minneapolis, MN), as described in methods. Mean age, blood glucose levels section and degree of insulitis development in each group are presented in Figure 4(b). The control group was characterized by much higher blood glucose levels (mean value = 28.38 mmol/L) and younger mean age (40.8 wks). Most of the surviving mice in the experimental groups had blood glucose sugar levels within the range of 12.9–14.07 mmol/L (mean values) and older mean age (113–114.5 wks). Although in many cases histological analyses showed a high percentage of insulitis (Figure 4(b)). In all vaccinated groups the synthesis of IL-1α (IL-1F1), IL-1β (IL-1F2), IL-3, IL-4, IL-7, IL-13, TNF-α (TNFSF1A), IL-1ra (IL-1F3), KC, JE (CCL12), MIP-1α (CCL3), and MIP-2 was reduced by 5.5–40% in comparison with the PBS control mice. In addition, in 2 of 3 treated groups the synthesis of IL-2, IP-10 (CXCL10), TIMP-1, sICAM-1 (CD54), and MIG (CXCL9) was reduced by 9–27%. In contrast, synthesis of IL-16 was higher by 17.5–19.3% in 2 of 3 treated animal groups as compared to PBS control. The level of the synthesis of IL-10 was reduced by 22.8 only in mice treated with rVV-CTB::GAD + CFA (1 : 10), but the level of IFN-γ was higher by 10.4%, or was reduced in other experimental groups by 9–19.6% in comparison with the PBS control mice. Synthesis of RANTES (CCL5) was reduced by 16.7% only in mice treated with rVV-CTB::GAD + CFA (1 : 0). Other cytokines/chemokines (data not shown) in other experimental groups were similar (Figure 4(a)). The level of cytokines IL-12, IL-5, and IL-6 was found to be approximately at background (data not shown). Analyses of the relationship between the percentage of the $CD3^+$, $CD4^+$, and $CD8^+$ cells of splenocytes show a low presence of $CD8^+$ cells as compared to $CD3^+$ and $CD4^+$ lymphocyte subsets (Figures 5(a) and 5(b)). There were significant differences between the percentage of $CD4^+$ T-cells in the gated lymphocyte population from mice inoculated with rVV-CTB::GAD + CFA (1 : 10) as compared to PBS and CFA (1 : 0) ($P = 0.027$ and 0.018, resp.; one-way Anova). However, there were no significant differences between the groups in the pattern of other T-cell subsets.

Analyses of the levels of proinflammatory, type 1 cytokine IL-12 and immunosuppressive, type 2 cytokine IL-10 in sera of animals showed a similar pattern ($P > 0.05$) in three analyzed treatment groups (naive, rVV-CTB::GAD + CFA (1 : 0), and rVV-CTB::GAD + CFA (1 : 10) (Figures 6(a), 6(b), and 6(c))). The mean value of IL-12 was significantly ($P < 0.05$) higher (52–56 pg/mL) in all experimental groups as compared to IL-10 (32–38 pg/mL). We did not find any correlation between cytokine levels in serums or in the collected samples with the age and the health status of the animals (data not shown).

We also analyzed the dynamics of VV-specific antibody production in vaccinated and control animal groups throughout the experiment to determine whether vaccination had a deteriorating effect ($P = 0.0004$) on the immune system in general (Figures 7(a) and 7(b)). The results of these analyses show that antibody production in mice inoculated with the control recombinant virus rVV-L15 and recombinant virus-vaccine rVV-CTB::GAD was similar and remained high over a long period of time, up to 60–80 wks of age.

4. Discussion

A multifunctional approach of systemic coinoculation of juvenile NOD mice with both rVV-CTB::GAD and CFA demonstrated a marked, synergistic decrease in hyperglycemia and pancreatic islet inflammation in comparison with the PBS control. The three treatment groups with the highest level of beneficial outcome demonstrate a gradual increase in hyperglycemia from week 13 through 61 weeks

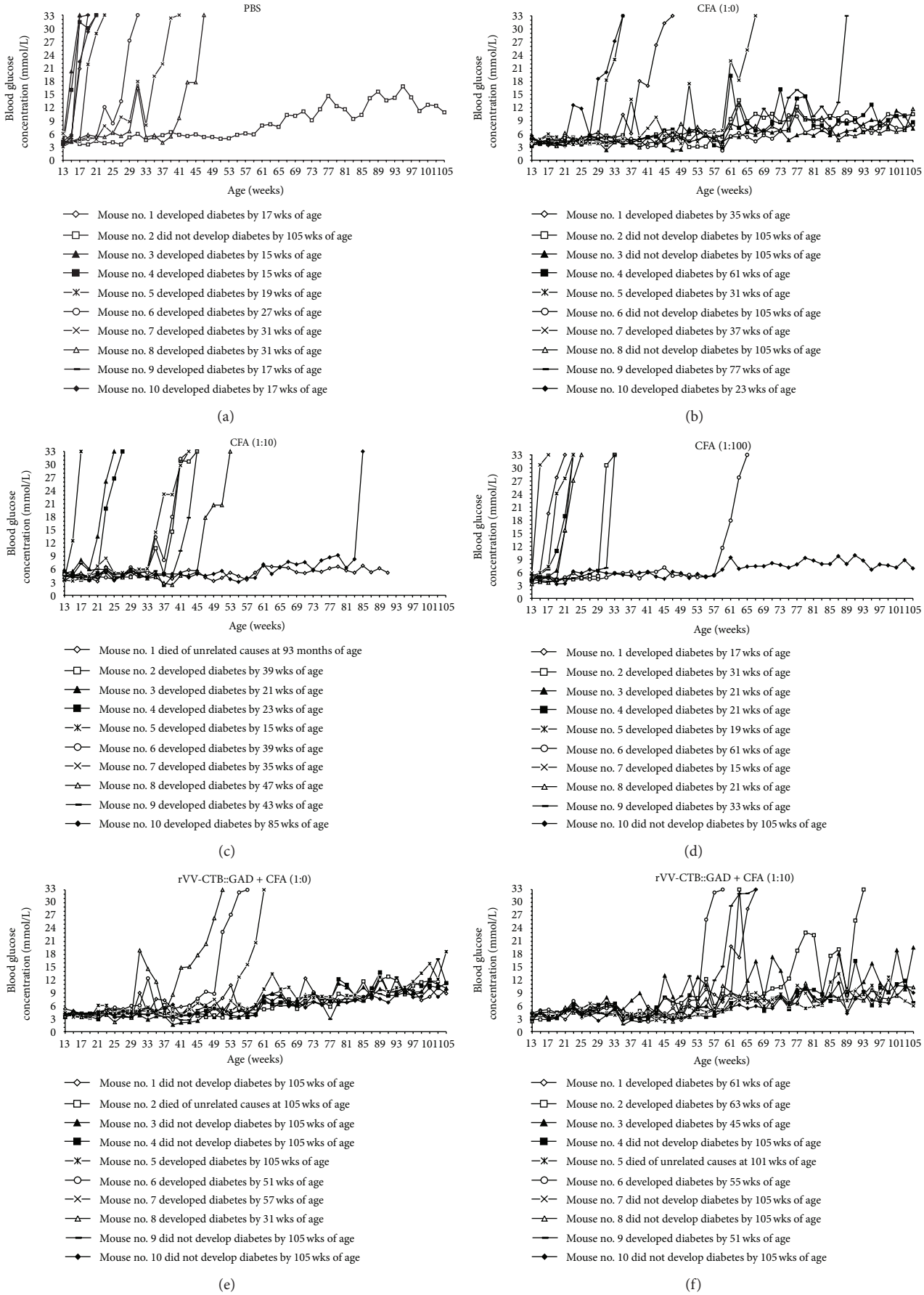


Figure 2: Continued.

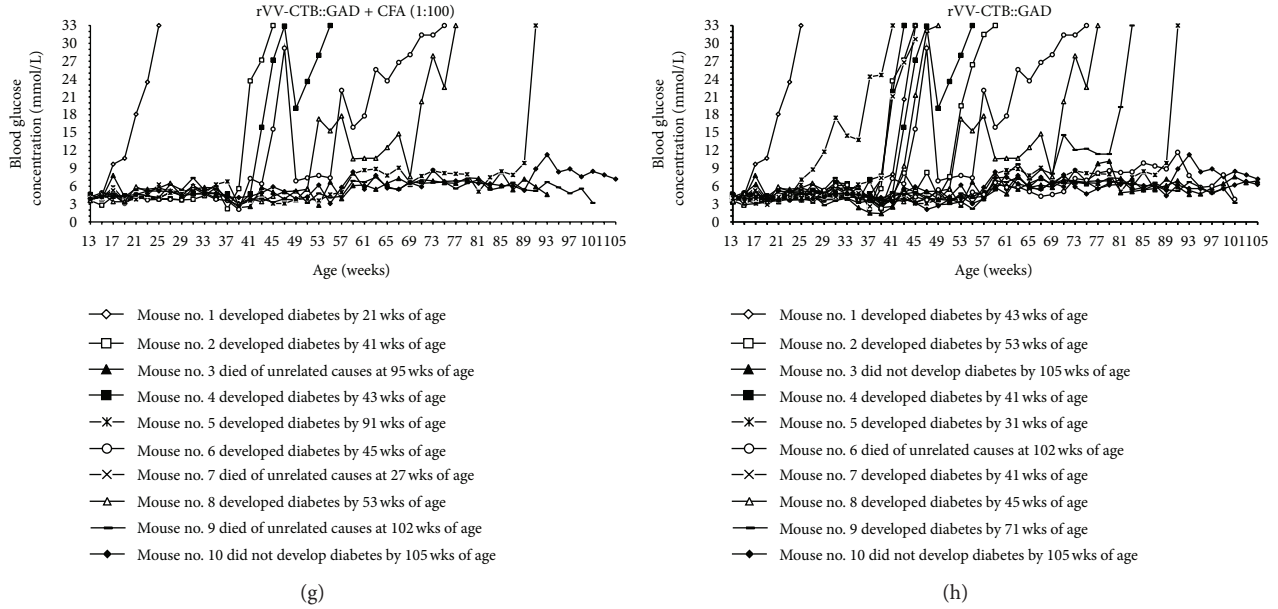
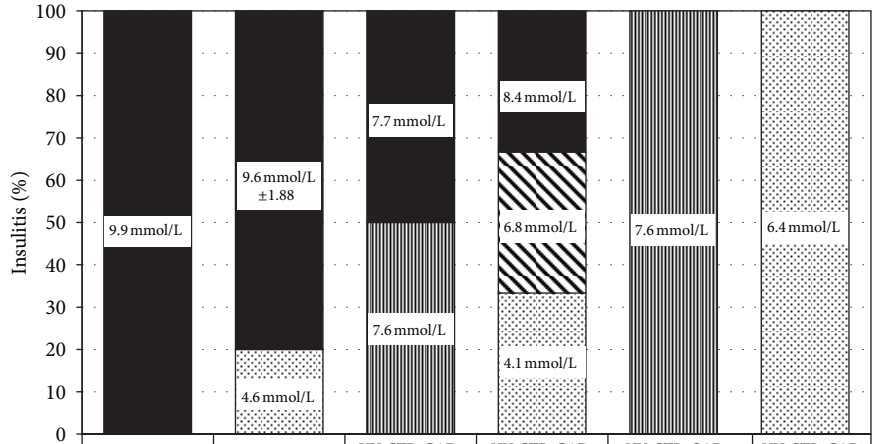
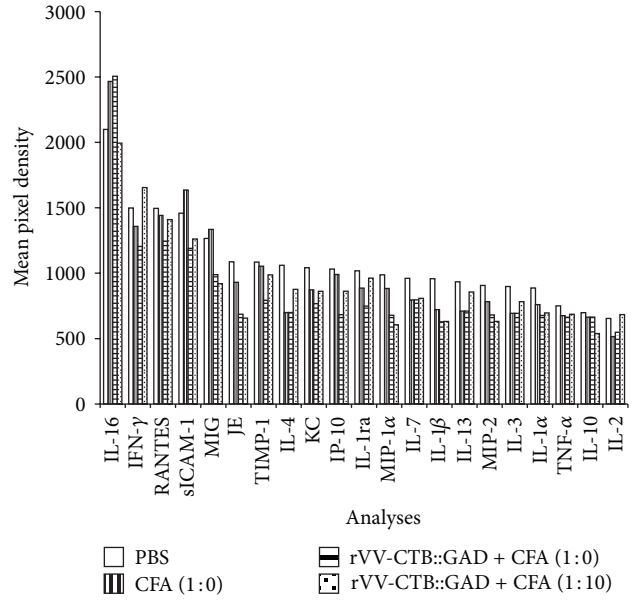


Figure 2: Blood glucose levels in individual animals of all experimental groups. (a) Mock-infected experimental group of 5-week-old NOD mice ($n = 10$) was inoculated with PBS. (b), (c), and (d) Three experimental groups of mice ($n = 10$) were subjected to s.c. injection with 0.1 mL of undiluted (1 : 0) and 1 : 10 and 1 : 100 diluted CFA. (e), (f), and (g) Groups of mice rVV-CTB::GAD + CFA (1 : 0), and rVV-CTB::GAD + CFA (1 : 10), rVV-CTB::GAD + CFA (1 : 100) ($n = 10$) were injected with the 0.3 mL virus (5×10^7 PFU/mL) i.p. and s.c. coinoculated with 0.1 mL CFA. (h) The rVV-CTB::GAD group of mice ($n = 10$) was subjected to i.p. injection with 0.3 mL of the virus (5×10^7 PFU/mL). Two weeks after the first injection, the vaccine inoculations were repeated. Beginning at 13 wks of age the individual mice in each experimental group were bled from the tail vein biweekly for 119 wks, and blood sugar levels were quantified.



	PBS ($n = 1$)	CFA (1 : 0) ($n = 5$)	rVV-CTB::GAD + CFA (1 : 0) ($n = 2$)	rVV-CTB::GAD + CFA (1 : 10) ($n = 3$)	rVV-CTB::GAD + CFA (1 : 100) ($n = 1$)	rVV-CTB::GAD ($n = 1$)
Score 4	1	4	1	1	0	0
Score 3	0	0	1	0	1	0
Score 2	0	0	0	1	0	0
Score 1	0	1	0	1	0	1
Score 0	0	0	0	0	0	0

Figure 3: Histological analysis of insulitis in mice at the end of the hyperglycemia study. Data on pancreatic tissue cross-sections of euglycemic mice of different treatment groups obtained from mice at 119 weeks of age are shown. Altogether, 13 mice (presented in the table below the graph) were analyzed and scored for insulitis: one mouse of the PBS control, rVV-CTB::GAD + CFA (1 : 100), and rVV-CTB::GAD treatment groups, five mice of the CFA (1 : 0), three mice of the rVV-CTB::GAD + CFA (1 : 10), and two mice of the rVV-CTB::GAD + CFA (1 : 0) groups. Blood glucose concentrations of individual (in mmol/L) or grouped (mean ± SD, in mmol/L) mice are also presented.



(a)

Group	Age (wk)	Blood glucose level (mmol/L)	Degree of insulitis (the infiltrated area %)
PBS	40.8 ± 19.6	28.4 ± 4.6	80.6 ± 12.1
CFA (1 : 0)	113 ± 5.8	12.9 ± 5.1	80.1 ± 16.9
rVV-CTB::GAD + CFA (1 : 0)	114.5 ± 2.7	14.6 ± 6.3	83.2 ± 5.9
rVV-CTB::GAD + CFA (1 : 10)	114.5 ± 4.5	13.1 ± 6.7	64.1 ± 19.6

(b)

Figure 4: Analyses of secreted cytokines/chemokines. (a) Cytokines/chemikines synthesized by splenocytes of CFA (1 : 0), rVV-CTB::GAD + CFA (1 : 0), and rVV-CTB::GAD + CFA (1 : 10) treatment group and naive (PBS) mice. (b) 4-5 mice from each experimental groups were euthanized and the synthesis of cytokines and chemokines in GAD-activated splenocytes was analysed. Means ± standard errors are shown for 4-5 mice/groups. Differences between ages of the treated groups of mice compared to PBS are statistically significant (one-way Anova, $P < 0.05$). Differences between groups in blood glucose and data of percentage of insulitis are statistically insignificant (one-way Anova, $P > 0.05$).

of age. Thereafter, the levels of hyperglycemia did not change significantly in these groups. In the two most effectively immunosuppressed groups, rVV-CTB::GAD + CFA (1 : 0) and rVV-CTB::GAD + CFA (1 : 10), immunological homeostasis was maintained through 29 wks and 43 wks, respectively. Thereafter, rapid increases in hyperglycemia in these groups were observed, suggesting the length of vaccine experiments with NOD mice can be followed to at least 60 wks of age. In many laboratories evaluation of T1D vaccine efficacy in NOD mice was limited for less than 30 weeks, providing less informative results. Based on analysis of this long-term study thus dramatic changes in diabetes symptoms in vaccine treated animal groups may be observed in animals between 30 and 63 weeks of age.

Overall, our experimental results clearly indicate that combinatorial vaccination with either tricomponent vaccines, rVV-CTB::GAD plus CFA complete (1 : 0) or diluted (1 : 10), results in a dramatic reduction in diabetes onset in NOD mice. In future animal studies we will investigate the therapeutic effects of the vaccine in NOD mice when the vaccine is applied during later stages of diabetes development, for example, 20–40 wks of age. These data may provide clues for resolving the important question of whether the CTB-GAD + CFA vaccine strategy can prevent T1D in prediabetic children more effectively than in children diagnosed with new-onset disease.

Immunotherapy with major islet β-cell antigens such as insulin, glutamic acid decarboxylase (GAD), or heat shock protein (hsp60), with or without immunomodulators, was shown to interfere with or prevent T1D onset [13–17]. Oral delivery of CTB conjugated with specific autoantigens was shown to protect mice against several Th1 cell-mediated autoimmune diseases including autoimmune encephalomyelitis [18], autoimmune chondritis [19], and uveitis [20]. Further, oral delivery of CTB-autoantigen conjugates were shown to suppress diabetes insulitis and hyperglycemia in NOD mice and several other animal autoimmune diabetes models [21, 22]. These experimental results

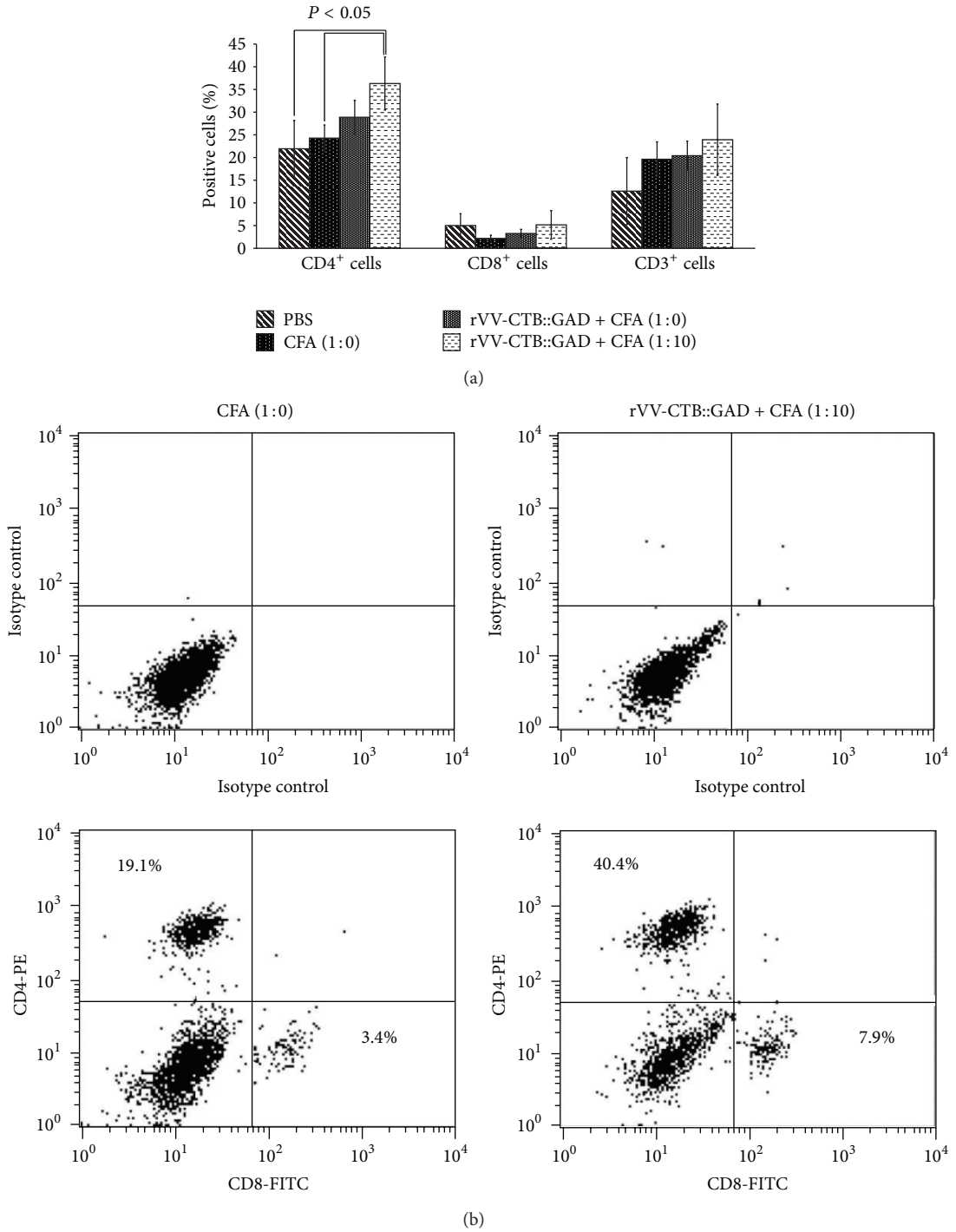


Figure 5: Analyses of splenocyte T-cell subsets. (a) Percentage of the $CD3^+$, $CD4^+$, and $CD8^+$ T-cell subsets in splenocytes of experimental mice measured by flow cytometry. The splenocytes of the hyperglycemic PBS ($n = 4$, median and average blood glucose 33 mmol/L, median age 21 wks, average 21.25 wks of age), euglycemic CFA (1 : 0) ($n = 4$ median 8.05 mmol/L, average 7.88 mmol/L, median/average 119 wks), rVV-CTB::GAD + CFA (1 : 0) ($n = 3$, median 7.7 mmol/L, average 7.77 mmol/L, median/average 116 wks), and rVV-CTB::GAD + CFA (1 : 10) ($n = 3$ median 6.8 mmol/L average 6.43 mmol/L, median 119 wks, average 117 wks) treated mice were analyzed after stimulation with GAD65. Differences between percentage of the $CD4^+$ T-cell subsets of the gated cell population of the mice inoculated with rVV-CTB::GAD + CFA (1 : 10) compared to PBS and CFA (1 : 0) are statistically significant ($P = 0.027$ and 0.018, resp.; one-way Anova). (b) Example of the relationship between the percentage of the $CD4^+$ and $CD8^+$ T-cell subsets of the gated cell population of the mice inoculated with CFA (1 : 0) and rVV-CTB::GAD + CFA (1 : 10). Cells were stained with antibodies for CD4 and CD8, as described in Section 2, and analyzed by flow cytometry. (The percentages of cells in quadrant, are shown relative to gated cells. Representative data from one mouse is displayed.)

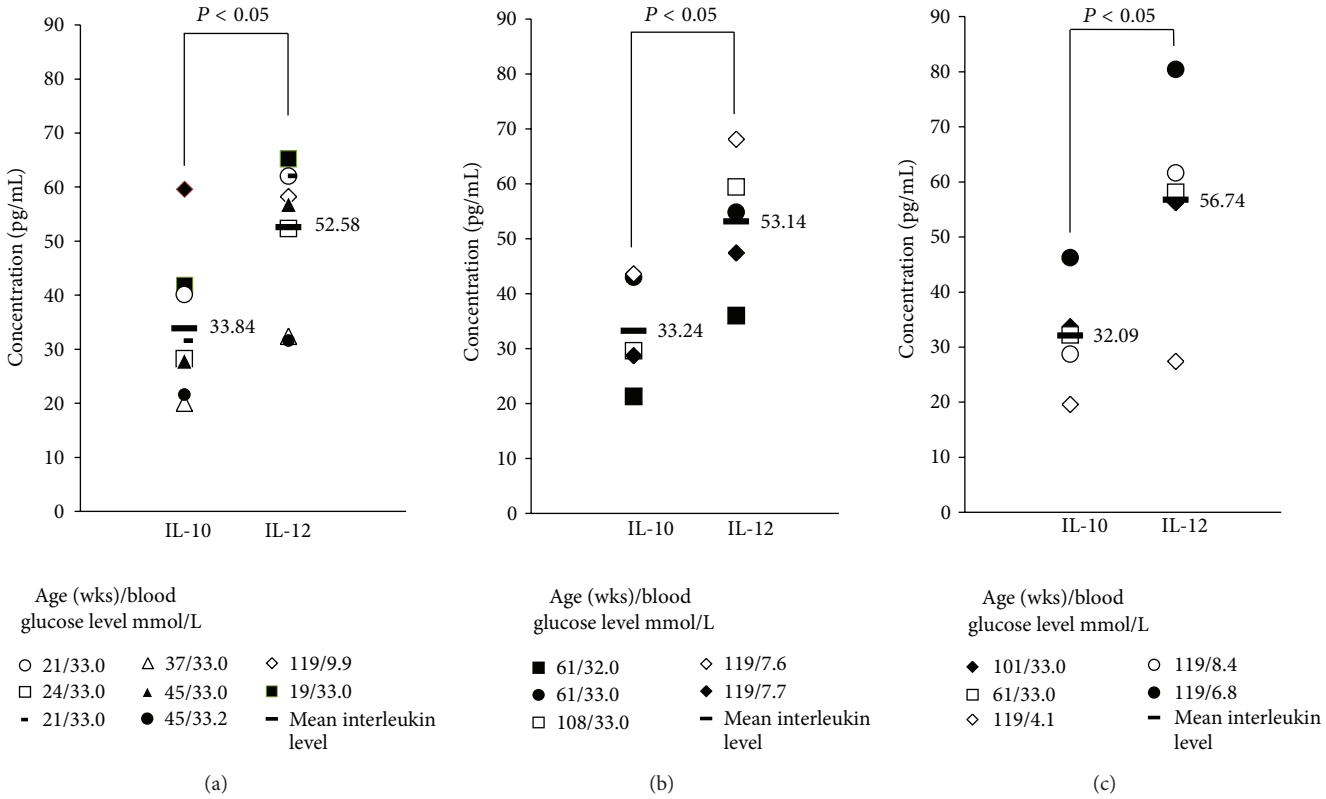


Figure 6: Detection of IL-10 and IL-12 in NOD mouse serum. (a) Secreted cytokines IL-10 and IL-12 in serum samples of the naive, (b) rVV-CTB::GAD + CFA (1 : 0), and (c) rVV-CTB::GAD + CFA (1 : 10) groups. Ages and blood glucose levels of individual mice are shown in columns on the right side.

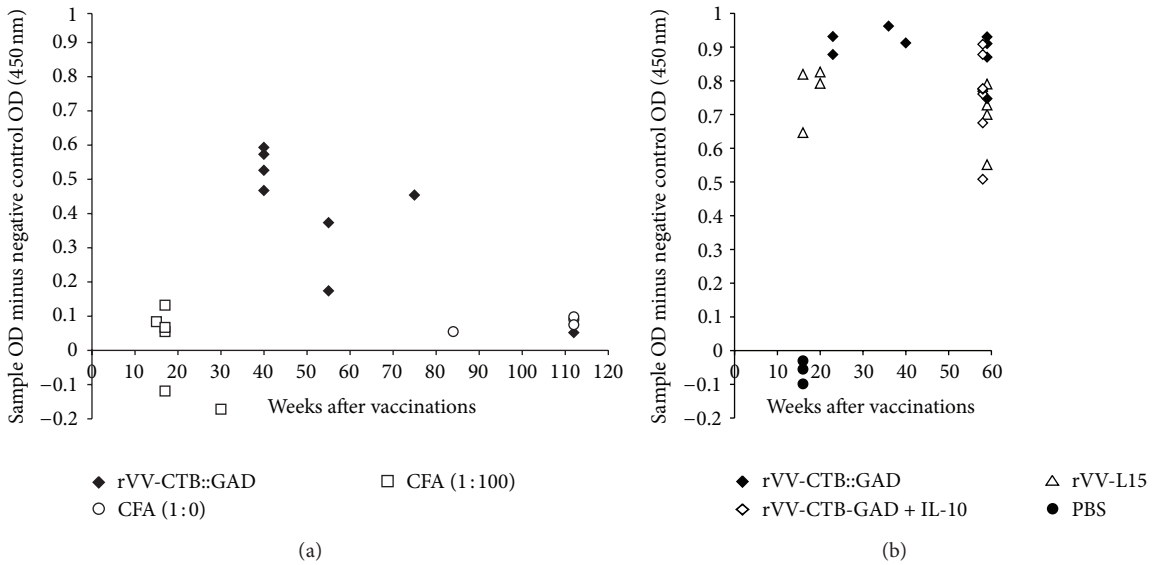


Figure 7: Vaccinia virus-specific humoral immune response in NOD mice. ((a) Exp. no. 1 and (b) Exp. no. 2). Determination of the VV-specific IgG was performed using indirect ELISA in two separate experiments. Naive (PBS), CFA (1 : 10), and CFA (1 : 100) mice served as controls for absence of VV-specific antibodies, while rVV-L15-treated [10] mice were controls for autoantigen-expressing virus rVV-CTB::GAD.

was associated with a reduction in inflammatory cytokine, IFN-γ production, and Tr1 regulatory T-cell migration into pancreatic islets [14, 23].

Previously, CFA and the closely related bacillus Calmette-Guerin (BCG), containing attenuated strain of *M. bovis* vaccines, were shown to modulate the development of T1D in animal models [24, 25]. Our experimental data confirm these previous experiments showing beneficial effects (60%) of CFA (1 : 0) and CFA (1 : 10) alone for prevention of T1D in NOD mice, which lasted until 59 and 37 weeks of age, respectively. An early study showed that the mycobacterial component of BCG serves as an immune potentiator of lymphocytes via TLR-mediated maturation of dendritic cells [26]. Administration of CFA was shown to prevent diabetes onset and to reduce the levels of insulitis in NOD mice [11]. In the KK-Ay mice studies, CFA vaccine provided controversial results [27]. In humans, BCG vaccination of T1D patients resulted in also conflicting data [25, 28]. Noteworthy, in currently ongoing clinical trials, D.L. Faustman expects that BCG will eliminate a population of disease-causing cells in T1D patients [29].

Immunotherapy with CFA was shown to be effective in preventing the spontaneous onset of autoimmune diabetes and in the restoration of self-tolerance to islet autoantigens [30, 31]. The protective effects of CFA were suggested to be mediated through the downregulation of autoreactive CTLs and the stimulation of NK cells. Most recent data however, demonstrate that CFA treatment ameliorates autoimmunity in NOD mice by up-regulating $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T-cells in pancreatic lymph nodes and for increasing TGF-β1 production [32], in spite of the fact that altered frequencies of peripheral $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T-cells were not yet shown to be specifically associated with type 1 diabetes [33].

Molecular mechanisms involved in autoantigen- and adjuvant-dependant immune suppression detected in NOD mice remain to be further elucidated. Evidence is available that CFA containing heat-inactivated *M. tuberculosis* cells induces T-cell-mediated immune responses, antibody production, and activation of the innate immune system [34]. Heat-shock protein-specific regulatory T-cells induced by mycobacteria may also contribute to CFA-induced suppression of diabetes [24]. Mycobacterial antigens may be presented to T-cells by different APC cells captured and presented by DC in NOD mice, sequestering capture and presentation of autoantigens. Protection may be attributable to an increase of antigen-presenting ability through maturation of DC and further that the mycobacterial cell wall skeleton is an essential adjuvant factor in CFA [26]. Complete Freund's Adjuvant was also suggested to induce clonal energy in effector cells that cause beta cell destruction [35].

An important issue linked to the adjuvants discussed here is their safety for human vaccine applications. Serendipitously, intravesical BCG was shown to be one of the most successful forms of immunotherapy in the treatment of human bladder cancer [36]. However, harmful effects, such as cystitis, dysuria, and haematuria, are often the main reason for discontinuing therapeutic treatment in many human studies. However, low-doses of BCG reduced these side effects without compromising therapeutic efficacy [37]. CFA was also the adjuvant of choice for immunization in laboratory animals for many decades. However, CFA has been associated with several local and systemic pathologies, including skin lesions and pneumonia [38]. Thus, CFA may cause significant side effects in humans, and lower doses may likely improve the safety features of the vaccine. In future studies, more precise dosage and frequency of CFA application will be established to allow the vaccine to be equally effective as well as safe for human vaccination applications.

Insulitis is thought to be associated with increased expression of proinflammatory cytokines (IL-1, TNF-α, and IFN-α) and type 1 cytokines IFN-γ, TNF-β, IL-2, IL-12 [39]. Lines of evidences suggest a role for chemokines in the pathogenesis of diabetes as well [40, 41]. Although previously we demonstrated that a combination of rVV-CTB::GAD and recombinant VV expressing the antiinflammatory cytokine IL-10 was effective in preventing the onset of T1D onset in NOD mice [10], we have not observed a significant increase in the synthesis of immunosuppressive IL-10 and IL-4 in any treatment groups of animals, as compared to PBS control. This experimental result is in agreement with a recent study of other authors [42]. In the sera of analyzed mice belonging to different treatment groups, we did not find changes in secreted IL-10 and IL-12 levels as well. Differences in the experimental results may be explained by difference in the environments where the individual treatments were performed. Our data confirm that multiple cytokines appear to participate in the autoimmune response that leads to β-cell destruction, and that deletion of a single "pathogenic" cytokine may not be sufficient to completely prevent diabetes development [39]. Although $CD4^+$ and $CD8^+$ T-cells are considered to be the primary mediators of β-cell destruction in NOD mice, our analyses of the relationship between the percentages of the $CD3^+$, $CD4^+$, and $CD8^+$ cells in splenocytes did not confirm this notion.

To determine whether vaccine therapy may impair normal immune function, immunity to foreign antigens was assessed in NOD mice following diabetes remission. Analyses of VV-specific humoral immune response following vaccination showed that the levels of specific antibody production in mice vaccinated with the control recombinant virus rVV-L15 or the recombinant virus-vaccine rVV-CTB::GAD were high and remained high during an extended period of time (60–80 wks of age). Thus, immunological tolerance against T1D autoantigens induced by our vaccine does not reduce or alter normal immune responses against foreign antigens (Figures 7(a) and 7(b)). From a safety perspective it is important to note that inoculation of NOD mice with the most effective T1D vaccine formulation rVV-CTB::GAD + IL-10 [10] did not impair the anti-VV humoral immunity of the vaccinated mice (Figure 7(a)).

Certain viruses, for example, EMC-D and KRV, were shown to be associated with the development of T1D in several animal models and in humans [5]. Other studies on infection with pathogens showed that certain viruses may have an opposite effect by ameliorating T1D disease in diabetic mice [43]. These earlier data prompted us [9] and others [44] to study prevention of T1D using vaccinia

viruses for delivery of genes encoding islet autoantigens. Jun et al. published experimental data claiming that VV expressing GAD may partially prevent diabetes in NOD mice [44]. Recombinant vaccinia virus experiments performed in our laboratory detected induction of an antiviral humoral immune response within 2 weeks following VV infection but did not show an effective VV-mediated or VV-GAD-mediated reduction of diabetes progression in NOD mice [10].

Vaccinia virus was shown to be a relatively safe and attractive virus vehicle for transgene delivery into a variety of mammals and humans for vaccination against infectious diseases [45]. To reduce potential side effects of live attenuated VV vaccines in immunocompromised individuals, we chose to use as a vaccine delivery vehicle the Lister vaccine strain of VV, which can be further attenuated by genetic manipulation [45]. Splenocytes from mock-infected mice secreted high levels of IFN-γ [10], whereas splenocytes isolated from mice inoculated with control VV secreted low to undetectable levels of the inflammatory cytokine IFN-γ. This result is in agreement with previous findings that VV infection of dendritic cells resulted in antigen-presenting cells that did not secrete inflammatory cytokines or initiate T-cell activation [46]. In contrast, VV expressing fused autoantigens, like insulin (rVV-CTB::INS) or GAD (rVV-CTB::GAD), could provide significant or moderate protection against development of T1D in NOD mice [9].

Here we demonstrated that systemic delivery of rVV expressing the immunostimulated autoantigen CTB::GAD, in combination with a normal or a 10-fold reduction in the dose of CFA, can confer a synergistic protective effect against diabetes onset in NOD mice. Further optimization of vaccine dosage may lead to more complete and safer protection of prediabetic NOD mice and humans against the progression of insulitis and hyperglycemia. An additional important goal of this study was to explore the duration of vaccine therapeutic effects. We found the majority of CTB::GAD + CFA-treated (1 : 0 and 1 : 10) mice remained euglycemic for more than a year up to 61–105 weeks of age, whereas most control mice developed hyperglycemia by 31 weeks of age. The levels of cytokines/chemokines were somewhat lower in the vaccinated mice, although the humoral immune system did not show signs of impairment. To better understand molecular mechanisms underlying the development of T1DM, therapeutic effects of this vaccination strategy will be assessed initially in NOD mice that have developed insulitis and hyperglycemia by 15–20 weeks of age. Moreover, the vaccine investigated in this study could be supplemented in the future with an additional viral construct expressing proinsulin (rVV-CTB::INS), which we demonstrated earlier to provide dramatic suppression of new diabetes onset in NOD mice [9]. The effectiveness of this multicomponent strategy for arresting or reversing the progression of insulitis and hyperglycemia in diabetic patients remains to be determined. Vaccinia virus-delivered immunomodulated autoantigens and CFA may provide continuous suppression of diabetes inflammation, thereby establishing an alternative approach for repopulation of the pancreas with "beta-like" insulin secreting cells, which could establish an effective and durable interventional therapy for restoration of euglycemia and immunological homeostasis in the large population of patients currently suffering from type 1 diabetes.

Conflict of Interests

All authors declare that they have no financial conflict of interests.

Acknowledgments

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Research Article

Chronic Deep Brain Stimulation of the Hypothalamic Nucleus in Wistar Rats Alters Circulatory Levels of Corticosterone and Proinflammatory Cytokines

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Deep brain stimulation (DBS) is a therapeutic option for several diseases, but its effects on HPA axis activity and systemic inflammation are unknown. This study aimed to detect circulatory variations of corticosterone and cytokines levels in Wistar rats, after 21 days of DBS-at the ventrolateral part of the ventromedial hypothalamic nucleus (VMHvl), unilateral cervical vagotomy (UCVgX), or UCVgX plus DBS. We included the respective control (C) and sham (S) groups ($n = 6$ rats per group). DBS treated rats had higher levels of TNF-α (120%; $P < 0.01$) and IFN-γ (305%; $P < 0.001$) but lower corticosterone concentration (48%; $P < 0.001$) than C and S. UCVgX animals showed increased corticosterone levels (154%; $P < 0.001$) versus C and S. UCVgX plus DBS increased IL-1β (402%; $P < 0.001$), IL-6 (160%; $P < 0.001$), and corsticosterone (178%; $P < 0.001$ versus 48%; $P < 0.001$) compared with the C and S groups. Chronic DBS at VMHvl induced a systemic inflammatory response accompanied by a decrease of HPA axis function. UCVgX rats experienced HPA axis hyperactivity as result of vagus nerve injury; however, DBS was unable to block the HPA axis hyperactivity induced by unilateral cervical vagotomy. Further studies are necessary to explore these findings and their clinical implication.

1. Introduction

The clinical use of deep brain stimulation (DBS) has increased in recent years [1]. This treatment has become a therapeutic option for pathologies that are associated with chronic pain and movement disorders [2] as well as for refractory depression [3] or epilepsy [4]. Such patients can be treated with direct electrical stimulation at the vagus nerve [5, 6] or at deep nuclei of the hypothalamus [4, 7–9]. The use of DBS in humans entails the implantation of a generator of electric current (commonly under the collarbone) and bilateral electrodes that transmit a continuous current to precise stereotaxic coordinates into the brain [10].

Although DBS was initially considered to mimic a lesion, the mechanism by which this therapy exerts its effects *in vivo* is complex and incompletely understood [11].

The electric stimulation of nerves triggers depolarization of the membrane in the associated neurons [12]. Accordingly, DBS devices induce axonal activation and neuronal inhibition in animal models [2, 13, 14]. Theoretically, these effects evoke activity in areas that received axonal projections that are adjacent to the stimulating electrode [15, 16]. The reported changes on neurotransmitters levels at anatomical area in which DBS is applied [17, 18] support this concept.

The hypothalamic nuclei are regions of interest to assess the interaction that exists between the nervous system and the immunological response since these hypothalamic nuclei anatomically connect two primary neural routes that modulate the inflammatory response: the HPA axis [19] and the sympathetic nervous system [20]. Additionally, both routes regulate the peripheral concentrations of the chief stress hormones cortisol, adrenaline, and noradrenaline [21].

The vagus nerve participates in a neural circuit that modulates innate immunity. This circuit is activated by cytokines and other inflammatory mediators in tissues that trigger afferent action potentials that travel by the vagus nerve. The ascending information is relayed to brainstem nuclei that control efferent neural signals that are transmitted back to the periphery in the form of action potentials via the vagus nerve [22]. This information is sent to the spleen and other cytokine-producing organs, where cytokine expression is inhibited by a molecular mechanism that requires the $\alpha 7$ subunit of the acetylcholine nicotinic receptor. The negative feedback by the motor arc of the inflammatory reflex prevents the damage of excessive innate immune responses—this circuit is known as the cholinergic anti-inflammatory pathway [22].

Until recently, no clinical or experimental study had described changes in vagus nerve function after application of DBS in hypothalamic nuclei. In epileptic patients, vagus nerve stimulation (VNS) reduces systemic levels of IL-6 and TNF-α but increases those of IL-10 and TGF-β [5, 23]. Such changes might be linked to its therapeutic effectiveness. Conversely, VNS elicits an anti-inflammatory response in several animal models of chronic and acute inflammatory syndromes [24–26]. VNS also regulates serum cortisol concentrations in patients [5] and corticosterone in rodents [27]. Vagal afferents represent a functional link between peripheral cytokine release and activation of the HPA axis. For example, subdiaphragmatic vagotomy blocks adrenocorticotropic hormone (ACTH) and corticosterone production when low doses of cytokines are administered intraperitoneally or intravenously [28–30]. However, activation of the HPA axis with higher doses of cytokines might involve additional neural and humoral pathways [28, 31, 32].

Activation of nerve fibers (i.e., once a nerve action potential is elicited) by chemicals or electrical stimulation establishes nerve-to-nerve or nerve-to-brain tissue communication. The solitary tract nucleus (STN)—the main terminal of vagal nerve afferents in the CNS—makes anatomic connections with corticotrophin-releasing cells in the paraventricular nucleus of the hypothalamus [33, 34]. Imaging studies have detected activation of the hypothalamus on electrical stimulation of the vagal nerve [35, 36]. Accordingly, Hosoi et al. reported elevation of serum corticosterone and ACTH on electrical stimulation of the vagal nerve in anesthetized rats [37]. These findings support a model in which electrical stimulation of the vagal nerve under experimental conditions activates brain structures that constitute the HPA axis. However, changes in the HPA axis or vagus nerve function due to DBS of hypothalamic nuclei have not been reported.

Our group hypothesized that electrical stimulation of hypothalamic nuclei during DBS would have immunoendocrine effects. Thus, our aim was to assess the immunological and endocrinological effects of chronic DBS (21 days) of the ventrolateral section of the ventromedial hypothalamic nucleus (VMHvl) in Wistar rats with or without unilateral cervical vagotomy (UCVgX). We measured the serum levels of corticosterone, IL-1β, TNF-α, IL-6, and IFN-γ in the absence of epileptogenic or antigenic stimuli.

2. Methods

2.1. Animals. Male Wistar rats, weighing 250–300 g at the time of surgery, were used. Animals were housed in individual cages at 25°C on a 12 h light/dark illumination cycle (light from 8 AM to 8 PM) and had free access to food and water. All animal procedures were performed as per the following guidelines: (i) the Neurology and Neurosurgery National Institute's Ethical Code for the care and use of laboratory animals, (ii) Mexican guidelines for the production, care, and use of laboratory animals (NOM-062-ZOO-1999), and (iii) the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications number 80-23, revised in 1978). All efforts were made to minimize animal suffering and reduce the number of animals that was used.

Animals were randomly assigned to one of the following experimental groups, each containing 6 rats: control without treatment (C), sham vagal surgery (S), unilateral cervical vagotomy (UCVgX), UCVgX plus electrodes without electric stimulation (UCVgX + E/WES), sham plus electrodes without electric stimulation (S + E/WES), DBS at VMHvl (DBS), and unilateral cervical vagotomy plus DBS (UCVgX + DBS). All animals were allowed to acclimate to the experimental conditions for 5 days before treatment.

2.2. Unilateral Cervical Vagotomy. Vagotomy was performed under general anesthesia with ketamine (80 mg/kg ip) and xylazine (5 mg/kg im) as described [38]. Briefly, a midline incision of 1.5 cm was made in the anterior neck to localize the right vagus trunk. The right vagus nerve was separated carefully from the carotid artery and cut, and the skin was sutured with mononylon. Sham (S) rats were operated on to expose the vagal trunk, but the vagus nerve was not cut. We performed unilateral cervical vagotomy to analyze the effects of partial blockage of vagal function.

2.3. DBS. Monopolar stainless steel electrodes of 100 μm, entirely insulated except for 0.25 mm at the tip (World Precision Instruments Inc., USA), were implanted bilaterally in anesthetized animals using a stereotaxic frame (Stoelting Co., USA) to reach the VMHvl (coordinates: 3.2 mm anterior,

0.6 mm lateral, and 9.6 mm ventral to the bregma) [39]. A 100 μm stainless steel electrode was placed on the skull bone as a reference. All electrodes were welded to a female connector. Anchor screws were set in the skull, and the assembly was secured with dental cement.

After surgery, the animals were allowed to recover in their home cage with food and water *ad libitum* and treated with buprenorphine (0.1 mg/kg ip) every 8 h over 3 days to minimize pain. After 1 week, animals were placed in acrylic boxes (30 × 30 × 30 cm) with a Plexiglas Arena and connected to a Grass S88 stimulator (Model PSIU6; Grass Quincy Mass, USA) by a flexible insulated cable that permitted free movement. Rats were allowed to explore the area for 30 min (habituation) before electrical stimulation with a fixed frequency of 50 Hz with an average current intensity of 550 μA for 30 s. The threshold of electrical stimulation was screened by increasing the current gradually until a change in behavior (sleep, excessive scratching, explorative behavior, sniffing, and ipsilateral ptosis) was observed, at which point current was fixed. VMHvl electrodes current intensity was adjusted for each animal to induce the change of behavior. The electrode which gave reliable behavioral response at a lower current intensity was chosen as the stimulating electrode for further experiments [40].

DBS treatment was administered during 30 minutes in which 30 cycles of alternate 30 s of electrical stimulation and 30 s of rest along 21 days were applied. Unstimulated control animals (WES) were connected to the stimulator without current being applied.

2.4. Quantification of IL-1β, TNF-α, IL-6, and IFN-γ by ELISA. After 21 days of treatment, blood samples (2.5 mL) were taken by cardiac puncture from anesthetized rats. Serum was separated and stored at −70°C until analysis. Rat cytokines were quantified using the ELISA Development System Kit and monoclonal antibodies against TNF-α (Cat. RTA00), IFN-γ (Cat. RIF00), IL-1β (Cat. RLB00), or IL-6 (Cat. R6000B) (all from R&D Systems, USA).

Antibodies were used to precoat a microplate before incubation with the samples (diluted 1 : 4) in triplicate. After wash steps, the appropriate enzyme-linked antibodies were added to the wells. Optical densities were quantified at 492 nm after addition of substrate and stop solutions. All incubations were performed at room temperature. Ranges of detection (pg/mL) were determined using standards as follows: IL-1β = 31.2–1000, TNF-α = 12.5–800, IL-6 = 62.5–2000, and IFN-γ = 31.2–1000. Intra- and interassay variability were less than 5% and 8%, respectively.

2.5. Quantification of Corticosterone by Radioimmunoassay. Total corticosterone from serum samples was quantified according to Keppler and Decker [41] in triplicate using the commercially available Coat-A-Count Rat Corticosterone radioimmunoassay (RIA) kit (Siemens) as per the manufacturer's instructions.

2.6. Statistical Analysis. Homogeneity of variance test for each molecule was followed by one-way ANOVA. Bonferroni's test was used as a *post hoc* test to compare responses between groups. Statistical analyses were performed using GraphPad Prism, version 6.00 for Mac OS X (GraphPad Software, USA). The statistical significance was established at $P < 0.05$.

3. Results

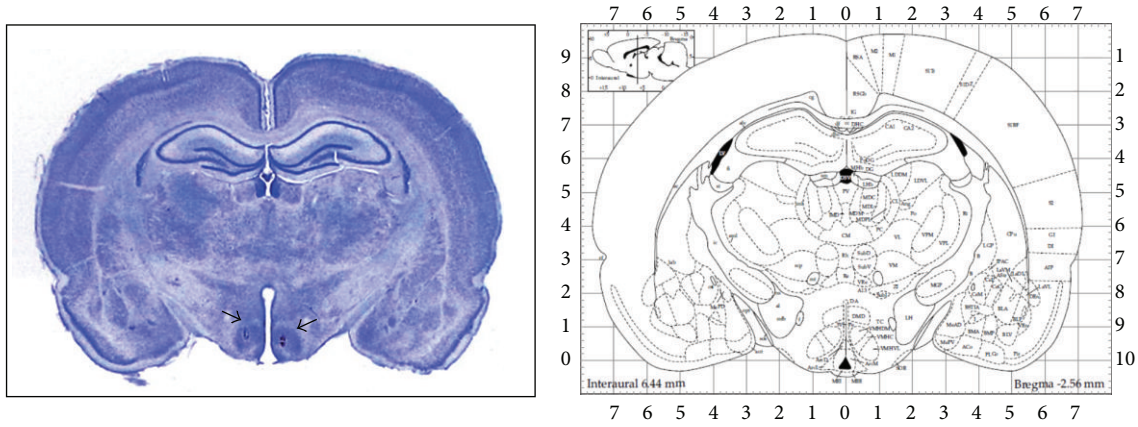
At the end of the experiments, localization of the electrodes in the VMHvl was confirmed with Nissl Technique-stained coronal sections (Figure 1). The circulatory concentrations of cytokines were quantified in serum from DBS-stimulated rats throughout the 21 days of treatment. All data are reported in pg/mL.

3.1. IL-1β. IL-1β serum concentrations differed significantly between groups ($F = 82.21$, df = 1,6; $P < 0.0001$). Unilateral vagotomy (UCVgX), DBS of the VMHvl (DBS), and their combination (UCVgX + DBS) increased IL-1β concentrations compared with the control (C) and sham (S) groups (55.5 ± 12, 56.5 ± 11, and 50.9 ± 11 versus 12.65 ± 1 and 12.21 ± 2, resp.; $P < 0.001$). There were no significant differences between UCVgX, DBS, and UCVgX + DBS animals. Levels in the sham, sham plus electrodes without electric stimulation (S + E/WES), and UCVgX plus electrodes without electric stimulation (UCVgX + E/WES) groups did not differ from those in the C group (Figure 2(a)).

3.2. TNF-α. TNF-α levels differed between treatments ($F = 82.21$, df = 1,6; $P < 0.0001$). The UCVgX and UCVgX + E/WES groups had lower levels compared with the C and S groups (13.7 ± 1 and 12.3 ± 1 versus 18 ± 2 and 17.02 ± 2; $P < 0.001$). Conversely, DBS increased TNF-α concentrations versus C rats (21.8 ± 2 versus 18 ± 2; $P < 0.001$). UCVgX + DBS rats had significantly higher TNF-α levels than UCVgX animals (22 ± 2 versus 13.7 ± 0.5; $P < 0.01$). Levels in S and S + E/WES rats did not differ compared with C rats (Figure 2(b)).

3.3. IL-6. IL-6 differed significantly between groups ($F = 23.11$, df = 6,1; $P < 0.0001$). IL-6 increased after UCVgX, DBS, and UCVgX + DBS treatments (79.8 ± 15, 83 ± 13, and 67.4 ± 12 versus 51 ± 6 in control rats; $P < 0.001$). UCVgX + E/WES reduced IL-6 levels compared with UCVgX (31.7 ± 3 versus 51 ± 6; $P < 0.01$). IL-6 after S and S + E/WES treatments was similar to levels in C rats (Figure 2(c)).

3.4. IFN-γ. IFN-γ levels differed significantly between treatments ($F = 120.8$, df = 6,1; $P < 0.0001$). DBS and UCVgX + DBS increased circulatory IFN-γ levels compared with C and S (115.5 ± 18 and 129.8 ± 13 versus 37.8 ± 6 or 40.5 ± 12; $P < 0.001$). Similarly, IFN-γ rose after UCVgX + DBS versus UCVgX (129.8 ± 13 versus 35.7 ± 2; $P < 0.001$). However, concentrations in UCVgX-treated rats were unchanged in S and C, indicating that DBS upregulates IFN-γ despite damage to the vagus nerve. Sham treatment had no effect versus C (Figure 2(d)).



(a) (b)

Figure 1: Sites of electrode implantation for cerebral electrical stimulation in rats. (a) Photograph of electrodes implanted in the ventrolateral portion of hypothalamic ventromedial nuclei, VMHvl (bregma −2.6 mm)in a coronal section of brain rat, stained with the Nissl Technique (5x). (b) Schematic representation of the VMHvl [39].

3.5. Corticosterone. Corticosterone levels differed between groups ($F = 28.97$, df = 6,1; $P < 0.005$). DBS-treated rats had lower concentrations than C animals (164.1 ± 11 versus 339.3 ± 31; $P < 0.001$). In contrast, UCVgX- and UCVgX + DBS-treated animals had higher levels than C or S rats (524.8 ± 2 and 606.6 ± 10 versus 339.3 ± 31 and 318.9 ± 8; $P < 0.001$). Groups C, S, and S + E/WES did not differ (Figure 3).

4. Discussion

The immune response cells constitutively express receptors for hormones, neurotransmitters, and cytokines [42], being susceptible to changes in the concentration of these soluble mediators. Our results show that the application of DBS or UCVgX leads to changes in circulatory levels of corticosterone and proinflammatory cytokines.

4.1. DBS Effects. A major issue influencing corticosterone and circulatory cytokine profiles reported in both treatments is the neuroendocrine, immune network. The HPA axis function is upregulated by proinflammatory cytokines through the brain receptors for these soluble molecules, expressed mostly at hypothalamus [43]. This stimulation induces a rise in circulatory levels of glucocorticoids that decreases the inflammatory systemic effects induced by cytokines and diminishes the release of CRH at hypothalamus, generating a negative feedback loop. In this study DBS and UVgX treatments induce an increase in circulatory levels of cytokines but only DBS treatment presents a significant decrease in corticosterone levels which are associated with functionality of HPA axis.

Serum glucocorticoid concentration is an accepted indicator of HPA axis activation [44]. As of the preparation of this paper, two studies in patients with Parkinson disease (PD) have described the effects of DBS at the subthalamic nucleus on the HPA axis. First, Novakova et al. reported significantly decreased cortisol levels from months 2 to 12 compared with baseline ($P < 0.01$, corrected) [45]. In the second report, Seifried et al. reported that 24 h mean cortisol levels decreased 6 months after electrode implantation surgery in PD patients (pre-OP 9.06 ± 2.63 versus post-OP 7.025 ± 3.46; $P = 0.05$) [46]. Similarly, de Koning et al. reported that obsessive-compulsive disorder patients that received DBS at nucleus accumbens showed a decrease of median urinary excretion of free cortisol [47]. Our results showing that Wistar rats that received chronic DBS at VMHvl had lower serum corticosterone levels are consistent with those reports.

The authors of those reports agree that DBS modulates HPA axis directly or indirectly through neural connections between the anatomical areas stimulated by DBS and the hypothalamus [45–47]. Moreover, Ballanger et al. reported that the effects of DBS are not restricted to a single anatomic location, since subthalamic nucleus DBS drives subthalamic nucleus output in not only the immediate target region but also the remote and widespread areas of the basal ganglia, brainstem, cerebellum, and cortex [48].

Our study shows that chronic DBS at VMHvl, which is a part of hypothalamus, limits activation of the HPA axis, reducing the levels of corticosterone. The HPA axis inhibition by DBS may be generated by neuronal blockage. Electrical stimulation depolarizes the membrane of neurons, inducing action potentials and triggering neurotransmitters release from vesicles. Under normal conditions, neurons have a period of rest that allows membranes to repolarize. However, chronic electric simulation affects the depletion of neurotransmitters, consequently impeding neuronal activation [49].

On the other hand, this is the first report to analyze the effects of DBS on systemic inflammatory responses. Our results demonstrate that chronic DBS at VMHvl increases serum levels of the proinflammatory cytokines IL-1β, TNF-α, IL-6, and IFN-γ. Unfortunately, the design of our study did not allow us to elucidate the mechanisms by which DBS induces systemic changes in proinflammatory mediators. We

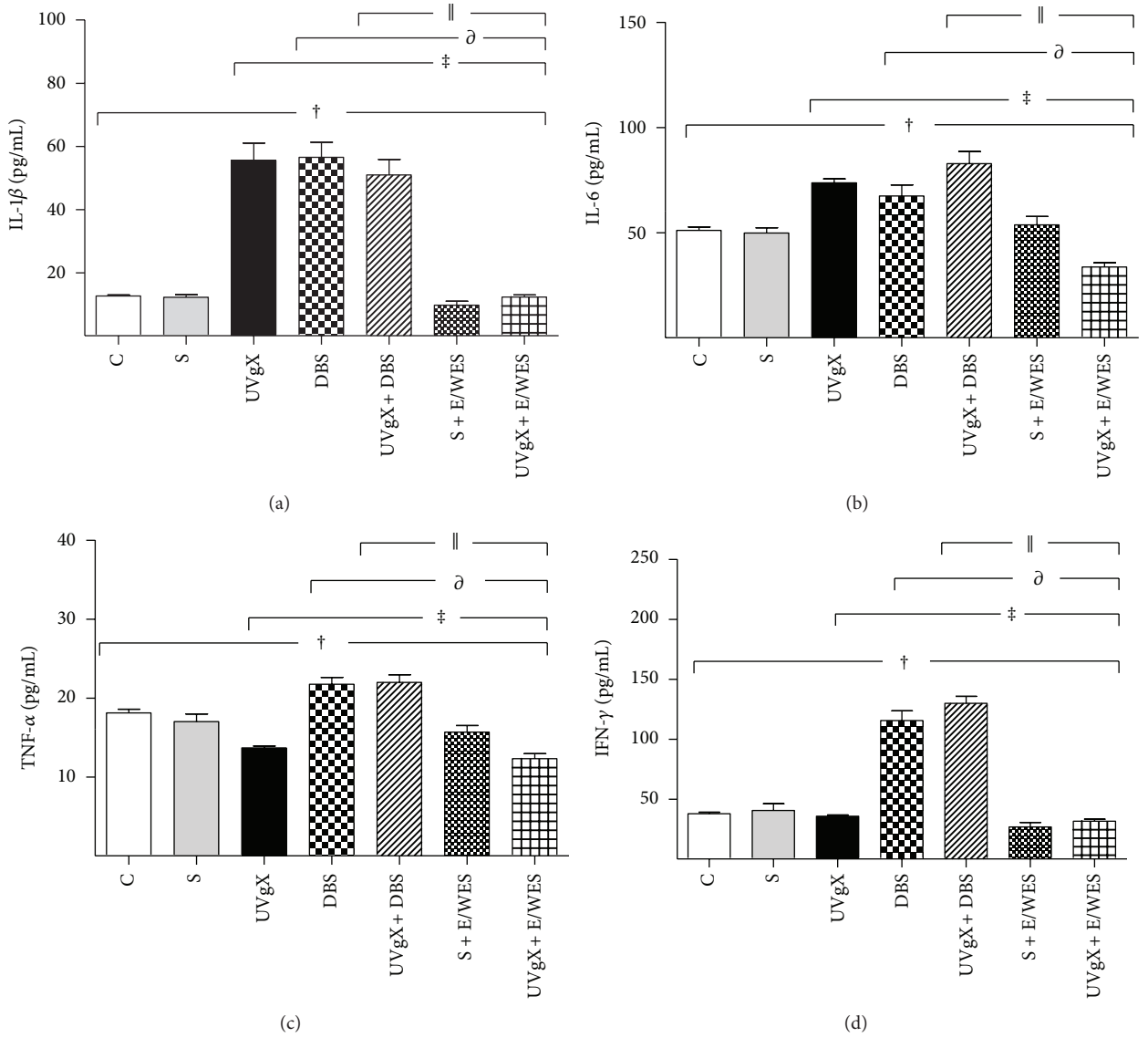


Figure 2: Effect of unilateral vagotomy and deep brain stimulation of hypothalamic nucleus on cytokine levels in Wistar rats. Serum IL-1β, IL-6, TNF-α and IFN-γ, levels were detected by capture ELISA assay. Animals received the following experimental procedures; Sham (S): animals were operated to expose the vagal trunk, without cutting the vagus nerve. Sham plus electrodes/without electric stimulation (S + E/WES). Unilateral vagotomy (UVgX): right vagus nerve was carefully separated from the carotid artery and cut. Unilateral vagotomy plus electrodes/without electric stimulation (UVgX + E/WES). Deep brain stimulation (DBS): animals were implanted bilaterally with two electrodes. Unilateral vagotomy plus deep brain stimulation (UV + DBS). Animals without treatment (C). Experimental groups (n = 6 rats) were followedup 21 days and showed significant differences compared to C, S, UVgX, DBS, and UVgX + DBS. Data are expressed as mean ± SE. The differences between means were evaluated with one-way ANOVA with Bonferroni's post hoc. Statistical significance was attributed when $P < 0.05$. $^{\dagger}P < 0.001$ C or S versus UVgX, DBS, UVgX + DBS, S + E/WES, and UVgX + E/WES groups; $^{\ddagger}P < 0.001$ UVgX versus DBS, UVgX + DBS, S + E/WES, and UVgX + E/WES groups; $^{\partial}P < 0.001$ DBS versus UVgX + DBS, S + E/WES, and UVgX + E/WES groups; $^{\|}P < 0.001$ UVgX + DBS, S + E/WES, and UVgX + E/WES groups versus C.

speculate that the significant decrease of corticosterone levels in rats that received chronically DBS promotes the establishment of proinflammatory profile of cytokines in circulation. Variations in glucocorticoids like corticosterone can modulate lymphocyte proliferation and cytokine gene transcription [50]. High levels of glucocorticoids compromise the function of immune response and promote an anti-inflammatory response; on the contrary low levels of glucocorticoids promote the release of proinflammatory cytokines [50].

4.2. UCVgX Effects. Unilateral cervical vagotomy (UCVgX + DBS and UCVgX) groups increased the circulatory levels of corticosterone compared with the rest of the groups, which

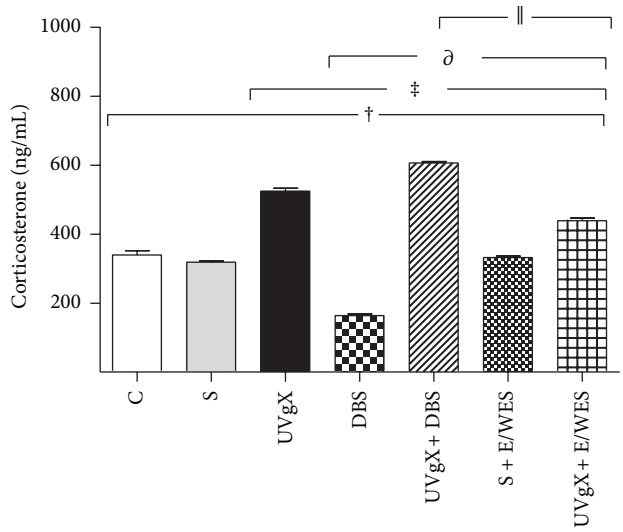


Figure 3: Effect of unilateral vagotomy and deep brain stimulation of hypothalamic nucleus on corticosterone levels in Wistar rats. Corticosterone determination was by RIA assay. Wistar rats received the following experimental procedures; Sham (S): animals were operated on to expose the vagal trunk, without cutting the vagus nerve. Sham plus electrodes/without electric stimulation (S + E/WES). Unilateral vagotomy (UVgX): right vagus nerve was carefully separated from the carotid artery and cut. Unilateral vagotomy plus electrodes/without electric stimulation (UVgX + E/WES). Deep brain stimulation (DBS): animals were implanted bilaterally with two electrodes. Unilateral vagotomy plus deep brain stimulation (UV + DBS). Animals without treatment (C). Experimental groups ($n = 6$ rats) were followedup 21 days and showed significant differences compared to C, S, UVgX, DBS and, UVgX + DBS. Data are expressed as mean ± SE. The differences between means were evaluated with one-way ANOVA with Bonferroni's post hoc. Statistical significance was attributed when when $P < 0.05$. $^{\dagger}P < 0.001$ C or S versus UVgX, DBS, UVgX + DBS, S + E/WES, and UVgX + E/WES; $^{\ddagger}P < 0.001$ UVgX versus, DBS, UVgX + DBS, S + E/WES, and UVgX + E/WES; $^{\partial}P < 0.001$ DBS versus UVgX + DBS, S + E/WES, and UVgX + E/WES; $^{\|}P < 0.001$ UVgX + DBS, S + E/WES, and UVgX + E/WES versus C.

is consistent with previous reports showing that cervical vagotomy in pigeons upregulated serum corticosterone [51] and that subdiaphragmatic vagotomy in rats intensified the carbachol (cholinergic muscarinic and nicotinic agonist) effects over ACTH and corticosterone secretion [52]. Apparently the effects of vagotomy on corticosterone levels may result from disruption of the motor fibers (parasympathetic control of target organs and perhaps immune cells) and/or the disruption of the sensory fibers (over 70% of the vagus is sensory) that carry information from periphery to the central nervous system including immune system derived signals. Disruption of sympathovagal balance in response to real or perceived challenges/stressors leads to alteration in homeostasis and activation of the HPA axis [53]. Our results show that unilateral cervical vagotomy did not reproduce the proinflammatory cytokine pattern that is observed in DBS group. This may be caused by the significant increase of corticosterone levels in rats with UCVgX.

4.3. UCVgX Plus DBS Effects. Both the HPA axis and the sympathetic nervous system regulate peripheral concentration of the main stress hormone cortisol [21]. Although the single effects of DBS and UCVgX produce opposite effects on serum cortisol concentration, the combined application of these treatments produces increases in corticosterone, IL-1β, and IL-6 levels. These results indicate that despite the existence of vagal afferents projects from brain steam to the solitary tract nucleus and hypothalamic nuclei, such as the VMHvl [33] DBS is unable to block the HPA axis hyperactivity induced by unilateral cervical vagotomy. This suggests that DBS neuronal blockage is not enough to reduce the anti-inflammatory response caused by UCVgX, because of either anatomic limitations on stimuli transmission or the existence of local compensation mechanisms.

UVgX and UVgX plus E/WES groups showed similar circulatory levels of corticosterone, TNF-α, and IFN-γ. Interestingly, the levels detected for IL-1β and IL-6 were reduced in UVgX plus E/WES in comparison to UVgX. These results seem to be paradoxical, albeit they suggest the possibility that other compensatory mechanism could be involved in these phenomena. In healthy individuals the levels of proinflammatory cytokines are controlled by several mechanisms, including the activation of the IL-6 receptor and gp130 protein. When IL-6 binds to its receptor, a mechanism is triggered by blocking Janus kinase signal and activator of transcription (JAK/STAT) mediated transcription of IL-1β [54], decreasing its level in circulation [55]. Additionally, the manipulation of the vagus nerve is associated with modification on food intake, body weight gain, HPA axis activation, and glucose metabolism [56], these metabolic changes may modify the circulatory levels of IL-1β and IL-6 [57].

4.4. Limitations. There were certain limitations in this study. First, the effects in DBS were evaluated in a small sample (each group with $n = 6$) of single rat strain. Second, circulatory levels of acetylcholine, adrenaline, and noradrenaline should have been measured to determine the contribution of the vagus nerve during DBS. Despite that in the present work there are not direct experimental line of evidence of the immunological or endocrinological effects of DBS, the following two facts should be considered: first, there is a general consensus about the communication between central nervous and immune systems which regulates several physiological processes [43]. In this study we did not explore the specific source of cytokines because they could come from many different sources. Leukocytes, neural cells, fibroblasts, adipocytes, and endothelial cells can all release cytokines and many of them constitutively express receptors to hormones and neurotransmitters [42]. Hormones and neurotransmitters might modulate the profile and circulatory levels of cytokines in this way. The second fact is that the general phenomenon described in this paper has been previously reported in other systems. The release of soluble mediators by HPA axis or vagus nerve activation, such as cortisol and acetylcholine, respectively, has direct effects on circulatory levels and profile of cytokines in animal models and patients, similar to those seen in this work. Lastly, we did

not perform a functional evaluation of the immune response, which would have required us to challenge the rats with infectious stimuli or cytokine administration during DBS or UVgX. Recent studies have reported that both the nature and the intensity of antigenic stimulation might affect the capability of hypothalamic nucleus to modulate HPA axis function, compared with the responses obtained during the stress stimulus [58]. All these issues will be considered in future studies.

5. Conclusions

Chronic DBS of the VMHvl impairs the HPA axis function, as reflected in the increase in circulatory levels of proinflammatory cytokines (IL-1β, TNF-α, IL-6, and IFN-γ) and decrease in corticosterone. UCVgX-treated rats experienced a HPA axis hyperactivity as a result of injury to the vagus nerve. DBS in UCVgX animals was unable to block the HPA axis hyperactivity induced by unilateral cervical vagotomy. These preliminary results suggest that immunity will be altered in patients who are treated with DBS, facilitating the development of strategies to prevent the secondary effects of DBS. Further studies are necessary to explore the clinical implications of these findings.

Conflict of Interests

All authors disclose that none has a commercial association that might pose a conflict of interests in connection with this paper.

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Research Article

PD-L1 Expression Induced by the 2009 Pandemic Influenza A(H1N1) Virus Impairs the Human T Cell Response

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PD-L1 expression plays a critical role in the impairment of T cell responses during chronic infections; however, the expression of PD-L1 on T cells during acute viral infections, particularly during the pandemic influenza virus (A(H1N1)pdm09), and its effects on the T cell response have not been widely explored. We found that A(H1N1)pdm09 virus induced PD-L1 expression on human dendritic cells (DCs) and T cells, as well as PD-1 expression on T cells. PD-L1 expression impaired the T cell response against A(H1N1)pdm09 by promoting $CD8^+$ T cell death and reducing cytokine production. Furthermore, we found increased PD-L1 expression on DCs and T cells from influenza-infected patients from the first and second 2009 pandemic waves in Mexico City. PD-L1 expression on $CD8^+$ T cells correlated inversely with T cell proportions in patients infected with A(H1N1)pdm09. Therefore, PD-L1 expression on DCs and T cells could be associated with an impaired T cell response during acute infection with A(H1N1)pdm09 virus.

1. Introduction

Programmed death-ligand 1 (PD-L1, B7-H1, CD274) is a coinhibitory molecule that has been associated with impairment of the T cell response. PD-L1 is one of the ligands that interact with the inhibitory PD-1 receptor, which is expressed on activated T cells [1]. PD-L1 expression is induced in a variety of human cells and tissues, including T cells and dendritic cells (DCs) [2]. PD-1/PD-L1 signaling interferes with the T cell response by blocking the CD28-mediated pathway, thereby affecting the expression of antiapoptotic genes, cell cycle progression [3], and cytokine production [4]. The role of the PD-1/PD-L1 signaling pathway in chronic infections, such as HIV or HCV infection, has been widely explored [5]. PD-L1 signaling is involved in the induction of T cell exhaustion, which impairs the response against pathogens. Additionally, this pathway is important in regulating the balance between an effective antimicrobial response and tissue damage [5]. The role of PD-1/PD-L1 during acute infections has been studied in mouse models of rabies [6], influenza [7], sepsis [8], RSV, and HMPV, and in patients with septic shock [9] with divergent findings, most of which suggest an inhibitory role for PD-L1. Recently, the expression of PD-1 and PD-L1 in the lungs of patients infected with the 2009 pandemic influenza A(H1N1) virus (A(H1N1)pdm09) was documented [10]. During chronic viral infections, PD-L1 expression on

T cells has been reported to be crucial in the impairment of the T cell response [5, 11]. However, PD-L1 expression on DCs and T cells during acute viral infections, particularly during A(H1N1)pdm09 infection, has not been widely studied.

Influenza virus infection may trigger an exacerbated immune response, which has been correlated with illness severity and sometimes death [12–14]. Lymphopenia is a clinical feature of influenza infections caused by seasonal influenza [15], avian H5N1 [16], and A(H1N1)pdm09 viruses [17]. With regard to the cellular immune response, leukocytes exposed to seasonal influenza virus have been shown to proliferate in response to the virus, but did not show a subsequent response to mitogen stimulation [18]. Additionally, influenza virus can induce apoptosis of several cell types, including peripheral blood-derived macrophages [19], avian cell lines [20], and T cells from healthy subjects [21].

Cellular immunity, may contribute to virus clearance, reduction of symptoms and prevention of secondary infections [22, 23]. The $CD4^+$ T cell-mediated immune response against influenza plays a role in limiting the severity of infection in the absence of previous antibodies [24]. However, during the acute phase of infection, T cells from patients infected with A(H1N1)pdm09 cannot differentiate into effector cells, highly express the death receptor CD95 (Fas), and do not respond to mitogens; nevertheless, T cell function is restored during the convalescent phase [25]. Therefore, the lymphopenia and T cell dysfunction reported in the A(H1N1)pdm09 infection might be induced by PD-L1 expressed on T cells, which could have affected T cell function through a mechanism similar to that which has been reported in chronic viral infections. This study evaluated the expression of PD-L1 on DCs and T cells and its effects on T cell response, as well as its possible implications during A(H1N1)pdm09 infection at the beginning of the 2009 pandemic outbreak at its epicenter.

2. Materials and Methods

2.1. Patients and Healthy Controls. Thirteen patients from two hospitals from the Mexican Social Security Institute (IMSS) with RT-PCR-confirmed pandemic influenza infection (pH1N1+), 12 PCR negative patients with influenza-like illness (ILI) (pH1N1−), and 10 healthy controls (HC) were included in this report. Patients were recruited during the first and second pandemic waves in Mexico City. Informed consent was obtained from participants. Study approval was obtained from the IMSS through the National Commission of Scientific Research, which comprises the Scientific, Ethics, and Biosafety Committees, in accordance with Good Clinical Practice. The project's ethics authorization number is: CNIC 2010-785-002.

2.2. Blood Samples and PBMC Separation. Blood samples from patients and controls were collected in EDTA tubes. Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) and cryopreserved until use. PBMCs from buffy coats were obtained from healthy volunteer donors according to institutional guidelines.

2.3. PBMC Stimulation. PBMCs (1×10^6) from buffy coats were placed in 24-well plates (Corning Inc., Corning, NY, USA) with RPMI-1640 (supplemented with HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 UmL^{-1} penicillin, and 100 μgmL^{-1} streptomycin, all from Gibco, Life Technologies, Carlsbad, CA, USA). They were stimulated with 10 $pgmL^{-1}$ staphylococcal enterotoxin B (SEB, Toxin Technology, Sarasota, FL, USA), 10 μgmL^{-1} of the TLR7 synthetic agonist CL264 (Invivogen, San Diego, CA, USA), 80 $HAUmL^{-1}$ (hemagglutination units) of live and UV-inactivated influenza A/Mexico/4482/2009(H1N1) virus and A/Panama/2007/1999(H3N2) virus provided by the Instituto Nacional de Referencia Epidemiológica (INDRE), or 10 $\mu gmL^{-1}$of recombinant A(H1N1)pdm09 virus hemagglutinin (HA), kindly provided by Dr. Clara Espitia from the Instituto de Investigaciones Biomédicas, UNAM. The PBMCs were incubated for 18 h, 3 or 7 days at 37°C/5% CO_2 prior to flow cytometry analysis of PD-1 and PD-L1 expression on DCs and T cells, respectively. For *de novo* protein synthesis analysis, PBMCs were stimulated with A(H1N1)pdm09 for 2 h, then cycloheximide (CHX, 50 μgmL^{-1}) was added to the culture for another 16 h.

2.4. T Cell and Dendritic Cell Enrichment and Culture. PBMCs (2×10^7) from buffy coats were incubated in supplemented RPMI-1640 at 37°C/5% CO_2 for 1.5 h, in Petri dishes (Fisher Scientific, Pittsburgh, PA, USA). Nonadherent cells were removed, washed, and quantified. T cells were then isolated by negative selection using a cocktail of PE-conjugated anti-CD19, anti-CD14 (eBioscience, San Diego, CA), anti-CD56, and anti-HLA-DR antibodies (BD Biosciences, San Jose, CA), with anti-PE magnetic microbeads in a MidiMACS system with LD columns (Miltenyi Biotec, Auburn, CA, USA). Dendritic cells were isolated the same way, but instead of anti-HLA-DR, PE-conjugated anti-CD3 (eBioscience) was used.

2.5. Stimulation of Enriched T Cells and DCs. The enriched T cells (5×10^5 cells/well) were placed into 48-well plates (Corning) with supplemented RPMI-1640 and stimulated with 10 $pgmL^{-1}$ SEB and 80 HAU/mL A(H1N1)pdm09 virus and incubated for 48 h at 37°C/5% CO_2. Enriched DCs (1.5×10^6) were placed in 24-well plates (Corning) with supplemented RPMI-1640 and stimulated with CL264 or A(H1N1)pdm09 virus. The cells were incubated for 18 h at 37°C/5% CO_2, collected, and labeled for flow cytometric analysis.

2.6. T Cell Proliferation and Cell Death. Buffy coat PBMCs (5×10^6 cells/well) were left untreated or stimulated with influenza virus and incubated for 18 h. Next, the cells were labeled with CFSE (Invitrogen Life Technologies, Carlsbad, CA, USA), and 5×10^5 cells/well were seeded into plates. These cells were left untreated or treated with 25 μgmL^{-1} anti-PD-L1 antibody 29E.2A3 (BioLegend, San Diego, CA, USA) or an isotype control MPC-11 (BioLegend) on days 0, 3, and 5, or with SEB (10 $pgmL^{-1}$) on day 0. The cells were incubated

for 7 days at 37°C/5% CO_2, collected, and proliferation was measured by flow cytometry. The proportion of apoptotic cells was detected by flow cytometry with Annexin V/Pacific Blue and 7-AAD staining (both from BioLegend).

2.7. Cytokine Production. Supernatants from the T cell proliferation culture were cryopreserved until use. Cytokine levels were measured using the human Th1/Th2/Th17 cytometric bead array kit (CBA) according to the manufacturer's instructions (BD Biosciences).

2.8. Sorting of cDCs and Isolation of $CD4^+$ Memory T Cells. Sorting of cDCs from enriched DCs was performed in a FACSAria cell sorter (BD Biosciences). After the preenrichment previously described, the negative cell fraction was labeled with anti-CD123-/PE-Cy5 and anti-HLA-DR/APC-Cy7 (BioLegend), to identify the cDC population; this population was isolated with a purity of about 90%. Memory $CD4^+$ T cells (T_m) were isolated with the human memory $CD4^+$ T cell isolation kit (Miltenyi Biotec). For co-culture assays, a 1 : 3 ratio of cDCs : T cells (1.5×10^4 cDCs and 4.5×10^4 T cells) were placed in 96-well plates with A(H1N1)pdm09 virus and incubated for 7 days at 37°C/5% CO_2, with or without PD-L1 blocking; the supernatant was collected to conduct CBA's analysis. Representative plots of cDCs and $CD4^+$ T cell purity are shown in Figure S1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2013/989673).

2.9. Flow Cytometric Analysis. The cryopreserved PBMCs from patients and controls were thawed and counted. Only the samples with total PBMCs above 1×10^6 cells were evaluated for PD-L1 expression on both DCs and T cells. When the number of cells was inferior, only DCs or T cells were analyzed. Hence, PD-L1 expression was analyzed on T cells of 9/13 pH1N1+ patients and 6/12 pH1N1− patients. In the case of DCs, PD-L1 expression was evaluated in 11/13 pH1N1+ patients and 10/12 in the pH1N1− group. The PBMCs from patients were labeled with the fluorochrome-conjugated antibodies, PD-L1/PE-Cy7, CD8/APC-Cy7, HLA-DR/APC-H7, CD4/PE-Cy7, CD123/PE-Cy5, and the lineage cocktail (Lin, CD3/PE, CD14/PE, CD56/PE, and CD19/PE) (BD Biosciences) and fixed with paraformaldehyde (4%). The T cells and DCs from buffy coats used for *in vitro* PD-L1 expression, proliferation, and cell death assays were labeled with all of the former antibodies, in addition to PD-1/FITC, CD4/APC-Cy7, CD2/PE, (BD Biosciences), and CD8/APC (Invitrogen). The cells were fixed with FACS Lysing Solution 1x (BD Biosciences) and cell viability was determined with Hoechst 33258 staining (Invitrogen). All samples were analyzed in either a FACSAria II or a FACSCanto II (BD Biosciences) using FlowJo (version 8.7) software (Tree Star Inc., Ashland, OR, USA). A minimum of 1×10^4 $CD2^+$ events were collected for T cell samples and 5×10^4 Lin-events for DCs samples.

2.10. Statistical Analysis. Statistics were calculated with Prism, version 5.0, from GraphPad Software (San Diego, CA, USA). To test for significant differences in PD-L1 expression between treatments, MFI values were normalized and a parametric Student's t-test with a two-tailed P value was performed. In the case of patients, a nonparametric Student's t-test was performed (Mann-Whitney test). Correlations were established with Spearman's test. Statistical significance was established at $P < 0.05$.

3. Results

3.1. PD-L1 Is Expressed on Human Dendritic Cells and T Cells, Whereas PD-1 Is Only Expressed on T Cells after Exposure to A(H1N1)pdm09 Virus. To test whether A(H1N1)pdm09 could induce PD-L1 and PD-1 expression on DCs and T cells, we stimulated human PBMCs with A(H1N1)pdm09. After 18 h of contact with the virus, we detected PD-L1 expression on conventional (cDCs) ($P < 0.01$) and plasmacytoid dendritic cells (pDCs) ($P < 0.001$) (Figure 1(a)). This was similar to the expression induced by the synthetic TLR7 agonist CL264. The A(H1N1)pdm09 virus induced PD-L1 on both $CD4^+$ ($P < 0.001$) and $CD8^+$ T cells ($P < 0.001$) similar to SEB (Figure 1(a)). We did not observe H3N2 seasonal virus induction of PD-L1 expression on any of the analyzed cells. In addition, after stimulation for 18 h with A(H1N1)pdm09 virus, we detected no PD-1 expression on DCs or T cells (Figure S2(a)). To evaluate whether PD-L1 expression on DCs could be related to viral infection, we stimulated DCs with live or UV-inactivated A(H1N1)pdm09 virus; we did not detect any significant differences in PD-L1 expression induced by live or UV-inactivated virus on DCs populations (Figure 1(b)), although it was slightly decreased in cDCs treated with the inactivated virus. Next, we considered the possibility that the kinetics of PD-1 and PD-L1 expression on T cells could be divergent; therefore, we analyzed the expression of these molecules over 7 days after A(H1N1)pdm09 stimulation; the highest expression of PD-L1 on both $CD4^+$ and $CD8^+$ T cells was detected after 18 h and decreased over time ($P < 0.001$) (Figure 1(c)). In the case of PD-1, we only observed significant differences after 3 days of virus stimulation in $CD4^+$ T cells and after 7 days in $CD8^+$ T cells ($P < 0.05$) (Figure 1(c)). To elucidate whether PD-L1 expression on DCs and T cells could be caused directly by interaction with the virus, we stimulated enriched DCs and $HLA\text{-}DR^+$ cell-depleted T cells with A(H1N1)pdm09. We found that PD-L1 expression on DCs was induced after interaction with the virus and was dependent on *de novo* protein synthesis (Figures 1(f) and 1(g)); however, PD-L1 expression induced on T cells by A(H1N1)pdm09 was dependent on the presence of APCs in the culture (Figure S2(b)), and on *de novo* protein synthesis (Figures 1(d) and 1(e)). When we stimulated PBMCs with A(H1N1)pdm09 virus for 2 h, and then added cycloheximide for 16 h, PD-L1 expression on both $CD4^+$ and $CD8^+$ T cells was inhibited. (Figures 1(d) and 1(e)). These results indicate that A(H1N1)pdm09 can induce PD-L1 expression directly on human DCs and in the case of T cells by *de novo* protein synthesis, albeit dependent on the presence of APCs as an early event. PD-1 expression on DCs was absent, and in the case of T cells, it was induced by A(H1N1)pdm09 later in time.

Table 1: Demographic data from patients.

Variable	Total patients ($n = 25$)	
	H1N1− ($n = 12$)	H1N1+ ($n = 13$)
Gender		
Female	5	5
Male	7	8
Age (years)		
Mean	46.8	34.4
Median	48.5	25.0
Max	76	78
Min	18	17
Leukocyte count (cells/mm^3) (mean ± SD)	7095.0 ± 4178.43	7643.8 ± 5122.75
Lymphocyte count (cells/mm^3) (mean ± SD)	1913.0 ± 1243.49	1403.8 ± 695.84

3.2. PD-L1 Signaling Impairs T Cell Response against Pandemic A(H1N1)pdm09 Virus. We analyzed if PD-L1 expression induced by A(H1N1)pdm09 could impair the T cell response against the virus. We blocked PD-L1 signaling during virus-induced T cell activation and established that blocking PD-L1 did not compromise T cell proliferation induced by the A(H1N1)pdm09 virus (Figures 2(a)–2(c)). However, we did observe that blocking PD-L1 decreased the proportion of early apoptotic (Annexin V$^+$ 7-AAD$^-$) CD8$^+$ T cells, 7 days after exposure to the H1N1 virus (Figures 2(d) and 2(f), $P < 0.05$). CD4$^+$ T cells did not show any significant differences in apoptosis after PD-L1 blocking (Figure 2(e)). We also determined that blocking PD-L1 increased PBMCs' production of IFN-γ, IL-10, and TNF. In order to evaluate if the virus caused this blocking effect, we stimulated the PBMCs with the recombinant hemagglutinin (HA) of the A(H1N1)pdm09 virus and measured cytokine production. We observed lower levels of IFN-γ, IL-10, and TNF when the whole A(H1N1)pdm09 virus was added, compared to HA stimulation. Moreover, when PD-L1 signaling was blocked, the cytokine levels induced by the virus were higher than those induced by the hemagglutinin. HA cytokine production was not affected by PD-L1 blockade, suggesting that this effect is A(H1N1)pdm09 virus-dependent (Figures 3(a)–3(c), $P < 0.05$). To evaluate if IFN-γ, IL-10, and TNF were mainly expressed by CD4$^+$ T cells, we co-cultured isolated memory CD4$^+$ T cells with sorted cDCs with or without PD-L1 blocking, and found that cytokine production by CD4$^+$ T cells was dependent on the presence of cDCs and increased when PD-L1 was blocked (Figures 3(d)–3(f), $P < 0.05$). Neither in bulk PBMCs nor in co-cultures of purified cells did we observe an effect of PD-L1 blocking on IL-4, IL-17A, or IL-6 production (data not shown). In addition, we found that after 7 days of culture, enriched cDCs still expressed PD-L1 after stimulation with A(H1N1)pdm09 virus, in contrast, high expression of PD-L1 was observed in memory CD4$^+$ T cells even in the absence of virus stimulation (Figure S3). Together, these results indicate that blocking PD-L1 on PBMCs had no effect on T cell proliferation but significantly decreased CD8$^+$ T cell apoptosis and increased IFN-γ, IL-10, and TNF production by CD4$^+$ T cells.

3.3. PD-L1 Expression Is Increased on Dendritic Cells and T Cells from PBMCs of Patients Infected with A(H1N1)pdm09 Virus. We evaluated PD-L1 expression in A(H1N1)pdm09 infection. We analyzed PD-L1 expression on T cells and DCs from cryopreserved PBMCs collected from patients during the 2009 influenza pandemic. Our study included 25 patients with influenza-like illness and 10 HCs, as shown in Table 1. Thirteen patients were RT-PCR positive for infection with the A(H1N1)pdm09 virus (pH1N1+), and the rest were categorized as pH1N1−. The median age of the patients was 48.5 years for H1N1− and 25 for H1N1+. Lymphopenia was present in most of the pH1N1+ patients (1403.8 ± 695.84 cells/mm^3) and in half of the pH1N1− patients (1913.0 ± 1243.49 cells/mm^3). We evaluated the frequencies and phenotype of DCs and T cells by flow cytometry; the gating strategy and representative plots are shown in Figure S4.

We found that pH1N1+ patients had a lower proportion of cDCs compared to the HCs (Figure 4(a), $P < 0.05$). No differences in the pDCs proportions between both groups were detected (Figure 4(b)). However, PD-L1 expression was increased on the cDCs and pDCs of both groups of patients compared to that of the HCs (Figure 4(c) pH1N1+, $P < 0.01$; pH1N1−, $P < 0.001$, and Figure 4(d), $P < 0.05$). CD4$^+$ T cells proportion tended to decrease in both groups of patients compared to that of HCs (Figure 4(e)). The CD8$^+$ T cell proportion was decreased in both groups of patients when compared to HCs (Figure 4(f), pH1N1+ $P < 0.05$, pH1N1− $P < 0.001$). Relative PD-L1 expression was increased on CD4$^+$ T cells in both groups of patients compared to that in HCs (Figure 4(g), pH1N1+ $P < 0.001$, pH1N1− $P < 0.01$), while in CD8$^+$ T cells, it was only increased in pH1N1+ patients (Figure 4(h), $P < 0.05$).

3.4. PD-L1 Expression on CD8$^+$ T Cells Is Associated with a Lower T Cell Proportion in Patients Infected with A(H1N1)pdm09 Virus. Finally, to establish if PD-L1 expression in PBMCs from patients could be associated with the T cell proportion during infection, we performed a series of correlations of DCs and T cell proportions and determined PD-L1 expression in pH1N1+ and pH1N1− subjects. We detected an inverse correlation between PD-L1 expression

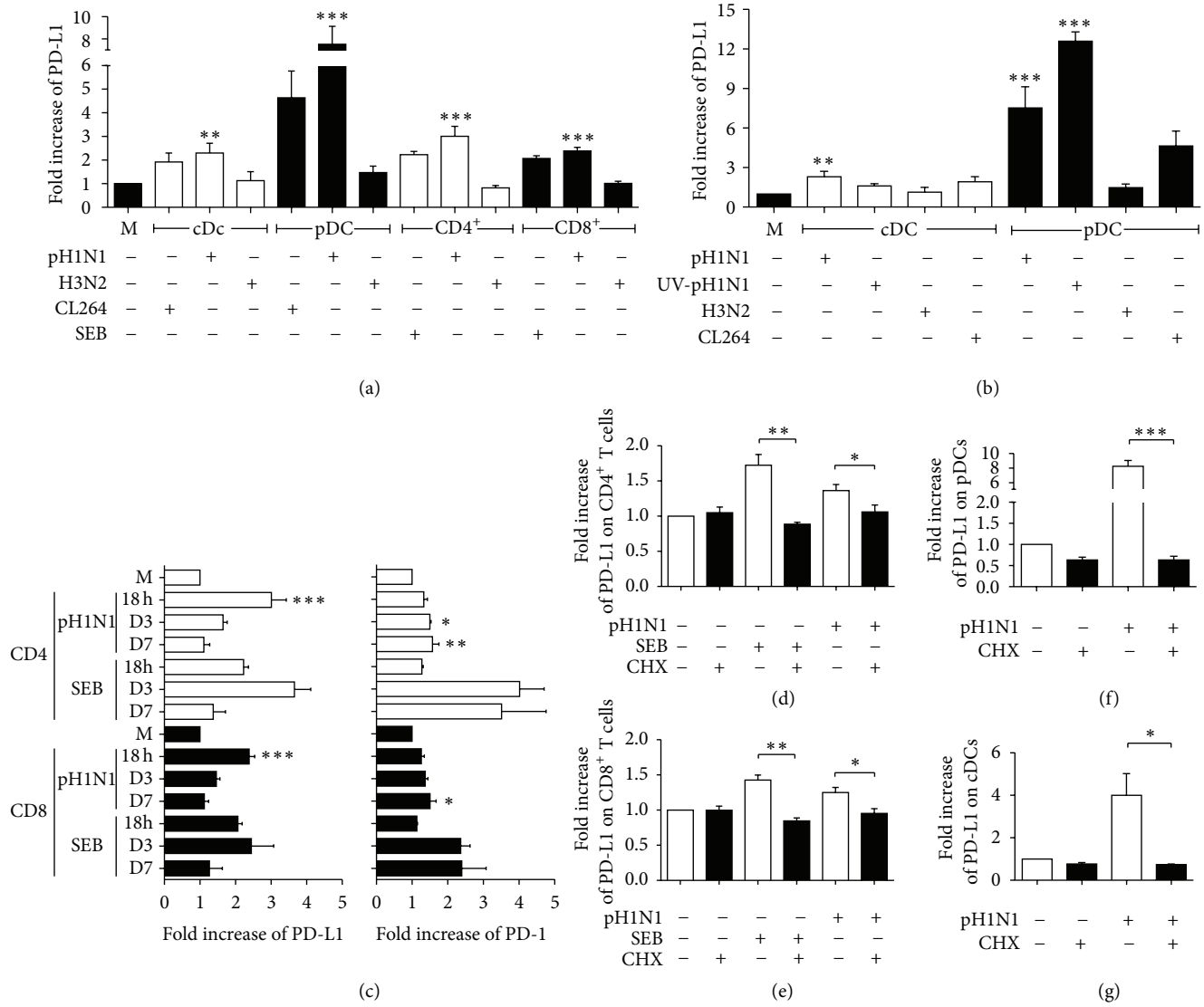

Figure 1: PD-L1 is expressed on human dendritic cells and T cells, whereas PD-1 is expressed only on T cells after exposure to A(H1N1)pdm09 virus. PBMCs were stimulated with A(H1N1)pdm09 virus (pH1N1), seasonal influenza virus (H3N2), staphylococcal enterotoxin B (SEB), or synthetic TLR7 agonist (CL264); PD-L1 and PD-1 expression on DCs and T cells was analyzed by flow cytometry. (a) Fold increase in PD-L1 expression on conventional (cDCs) and plasmacytoid dendritic cells (pDCs), $CD4^+$, and $CD8^+$ T cells after 18 h of stimulus. M: medium. (b) PBMCs were stimulated with live or UV-inactivated pH1N1 for 18 h; virus and PD-L1 expression was measured on cDCs and pDCs. (c) Kinetics of PD-L1 and PD-1 expression on $CD4^+$ and $CD8^+$ T cells induced by pH1N1 or SEB. PBMCs were stimulated with pH1N1 for 2 h, then cycloheximide (CHX) was added for another 16 h, and PD-L1 expression on $CD4^+$(d), $CD8^+$ T cells (e), pDCs (f), and cDCs (g) was measured by flow cytometry. ($n = 5$ donors, error bars indicate standard error of the mean (SEM)). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by one way ANOVA test with Bonferroni posttest.

on $CD8^+$ T cells and the proportion of both T cell subsets only in pH1N1+ patients (Figures 5(a) and 5(b), $P < 0.05$); we did not find a significant correlation between PD-L1 expression and cell proportion in pH1N1− subjects or in DCs subsets (data not shown). As a whole, these results suggest that PD-L1 expression on T cells could be one of the factors mediating the decrease in the T cell proportion in pHN1+ patients.

4. Discussion

PD-L1 expression plays a critical role in chronic infections by impairing T cell function [5]. We report here that PD-L1 expression on DCs and T cells impairs T cell response to the influenza A(H1N1)pdm09 virus *in vitro*. We also suggest that PD-L1 expression could have implications during the acute natural infection.

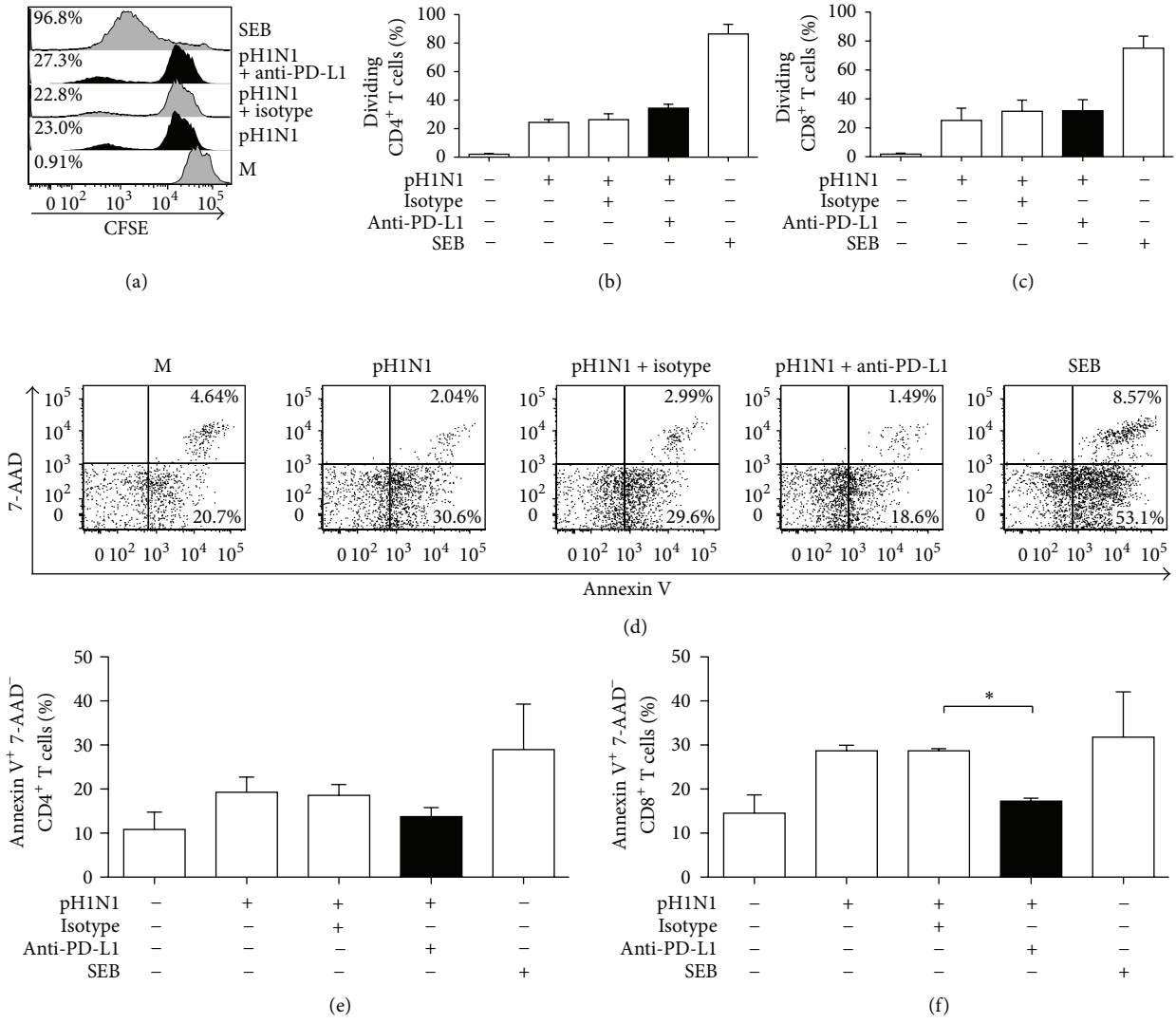

Figure 2: PD-L1 signaling blockade decreased $CD8^+$ T cell death *in vitro* but did not have an effect on T cell proliferation in response to A(H1N1)pdm09 virus. PBMCs from healthy individuals were stimulated with A(H1N1)pdm09 for 18 h, washed, labeled with CFSE, and treated on days 0, 3, and 5 with a blocking anti-PD-L1 antibody or an isotype control. Cells were incubated for 7 days, and T cell proliferation and cell death were determined. SEB was used as a control. (a) Representative histograms of the $CD4^+$ T cell CFSE dilution from one individual. (b, c) T cell proliferation expressed as the percentage of $CFSE^+$ dividing cells. (d) Representative plot of Annexin V and 7-AAD staining to evaluate $CD8^+$ T cell apoptosis, which was gated from $CD2^+$ and $CD8^+$ cells. (e, f) Percentage of early apoptotic (Annexin V^+ 7-AAD^-) T cells. ($n = 7$, error bars indicate SEM). $^*P < 0.05$ by Student's t-test.

A(H1N1)pdm09 was able to induce PD-L1 expression on DCs in a similar manner to a TLR7 ligand. It has been documented that TLR7 and retinoid-induced gene receptor 1 (RIG-1) mediate the recognition of influenza virus in DCs [26]. Therefore, human peripheral DCs may recognize the A(H1N1)pdm09 influenza virus through these receptors and subsequently express PD-L1 through a mechanism similar to that reported in influenza and other viral infections [6, 27]. PD-1 and PD-L1 expression can be induced on T cells through TCR signaling [2, 28]. We found that the PD-L1 expression induced by A(H1N1)pdm09 on T cells was APC-dependent, and mostly hinged on *de novo* protein synthesis. Additionally, we show that H3N2 seasonal virus failed to induce PD-L1 expression on either DCs or T cells; considering UV-inactivated pH1N1 induced PD-L1, it is possible that the expression of PD-L1 observed is independent of the infection capacity of the viruses. According to these results, we conclude that *in vitro*, there is an important difference between the pandemic and the seasonal influenza viruses in terms of their ability to induce PD-L1 expression. Furthermore, we found that PD-1 up regulation was detected at late time points in $CD4^+$ T and $CD8^+$ T cells cultures, after contact with the A(H1N1)pdm09 virus. This result is concordant with those observed in a recent mouse infection model, in which

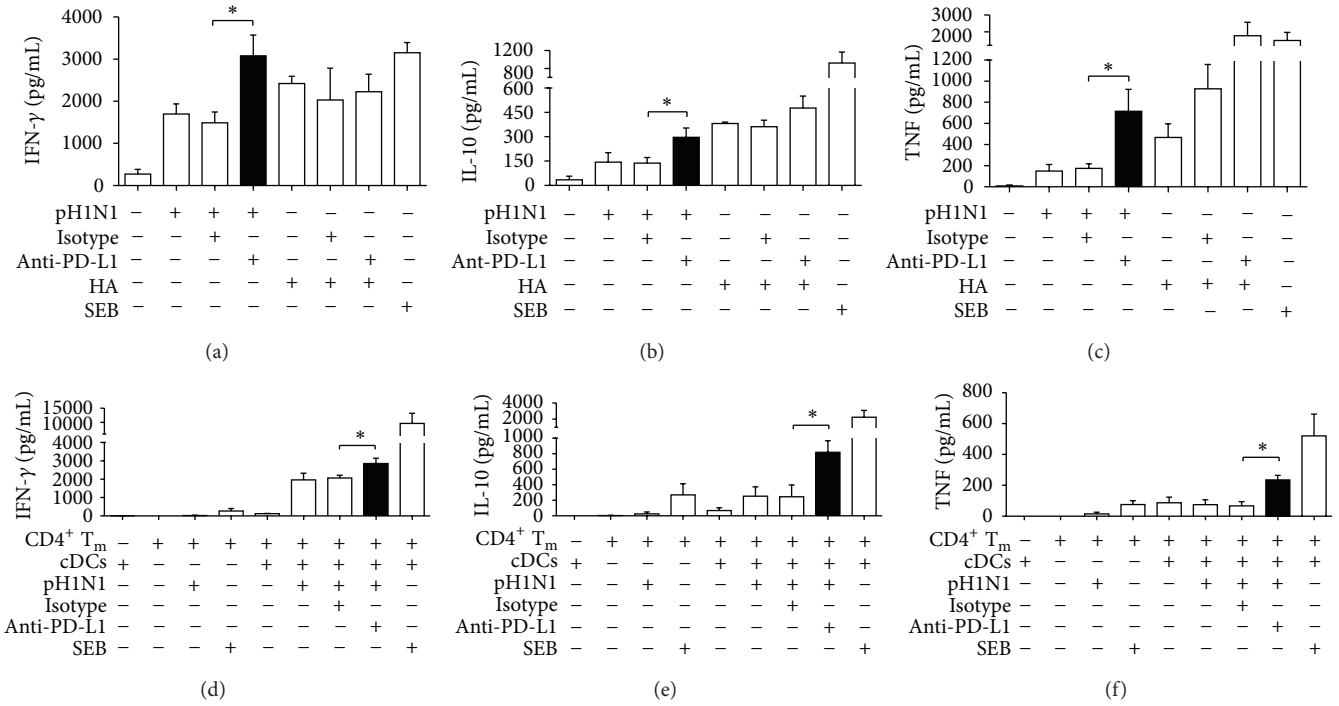

Figure 3: PD-L1 blocking increased *in vitro* IFN-γ, IL-10, and TNF production, predominantly by $CD4^+$ T cells in response to A(H1N1)pdm09 virus. Cytokine levels in the supernatants (SN) of PBMCs cultured for 7 days as described in Figure 2 and PBMCs stimulated with hemagglutinin (HA) for 7 days were measured with a Th1/Th2/Th17 human cytometric bead array kit (CBA). The production of IFN-γ (a), IL-10 (b), and TNF (c) by PBMCs is shown. Isolated memory $CD4^+$ T cells (T_m) and sorted cDCs were co-cultured with or without PD-L1 blocking for 7 days, and cytokine production in the SNs was measured; IFN-γ (d), IL-10 (e), and TNF (f) levels are shown. Results are duplicates from 3 independent experiments and error bars indicate SEM. pH1N1: A(H1N1)pdm09 virus; SEB: staphylococcal enterotoxin B. $^*P < 0.05$ by Student's t-test.

cognate viral antigen was necessary and sufficient to induce PD-1 expression on T cells, and that PD-1 was expressed by lymphocytes in the lower airways during acute influenza infection in humans [10].

PD-L1 expression has been associated with T cell exhaustion and dysfunction during chronic viral infections and in some acute infections in both *in vitro* and *in vivo* models [6, 27]. We have shown that similar T cell impairment mechanisms might also develop after interaction with A(H1N1)pdm09. Blocking PD-1/PD-L1 interaction enhanced the T cell response against A(H1N1)pdm09 virus. Apoptosis was significantly decreased in $CD8^+$ T cells, whereas cytokine production was increased; however, no impact was observed on T cell proliferation. One explanation for these results could be the late up regulation of PD-1 expression on T cells. Therefore, T cell proliferation may not be affected by the PD-L1 blockade because PD-1 expression was not apparent until day 3; however, the effects that we observed on $CD4^+$ T cell differentiation (cytokine production) when PD-L1 signaling was blocked, could be attributed to their expression of PD-1 until day 3. In addition, PD-L1 expression is maintained over time (7 days) in A(H1N1)pdm09 stimulated cDCs and is highly expressed on memory $CD4^+$ T cells, indicating that these cells could be a source of PD-L1 during the late phase of T cell differentiation. Moreover, PD-1 is expressed on $CD8^+$ T cells 7 days after A(H1N1)pdm09 stimulation, which correlates with decreased T cell death. There are previous reports suggesting that after direct virus exposure, human $CD8^+$ T cells are more susceptible to apoptosis than $CD4^+$ T cells [21]. In agreement with this finding, we observed that blocking PD-L1 after stimulation with A(H1N1)pdm09 could prevent $CD8^+$ but not $CD4^+$ T cell death.

Interestingly, we also observed that blocking PD-L1 caused an increase in the production of IL-10, IFN-γ, and TNF that may be associated with impairment of T cell differentiation induced by the virus, because when we stimulated cells with HA and blocked the PD-1/PD-L1 interaction, we did not observe any effects on cytokine production. The A(H1N1)pdm09 virus has also been reported to induce a decrease in cytokine levels in human DCs when compared with seasonal viruses *in vitro* [29]. Our study shows that PD-L1 expression induced by A(H1N1)pdm09 could inhibit the production of both inflammatory and regulatory cytokines in human bulk PBMCs and in co-cultures of purified cDCs and memory $CD4^+$ T cells. It has been established that T cells from patients infected with A(H1N1)pdm09 cannot differentiate into effector cells, do not respond to mitogens, and highly express CD95 (Fas), suggesting an apoptosis-related mechanism for the lymphopenia reported in A(H1N1)pdm09 infection [25]. Furthermore, these findings could contribute

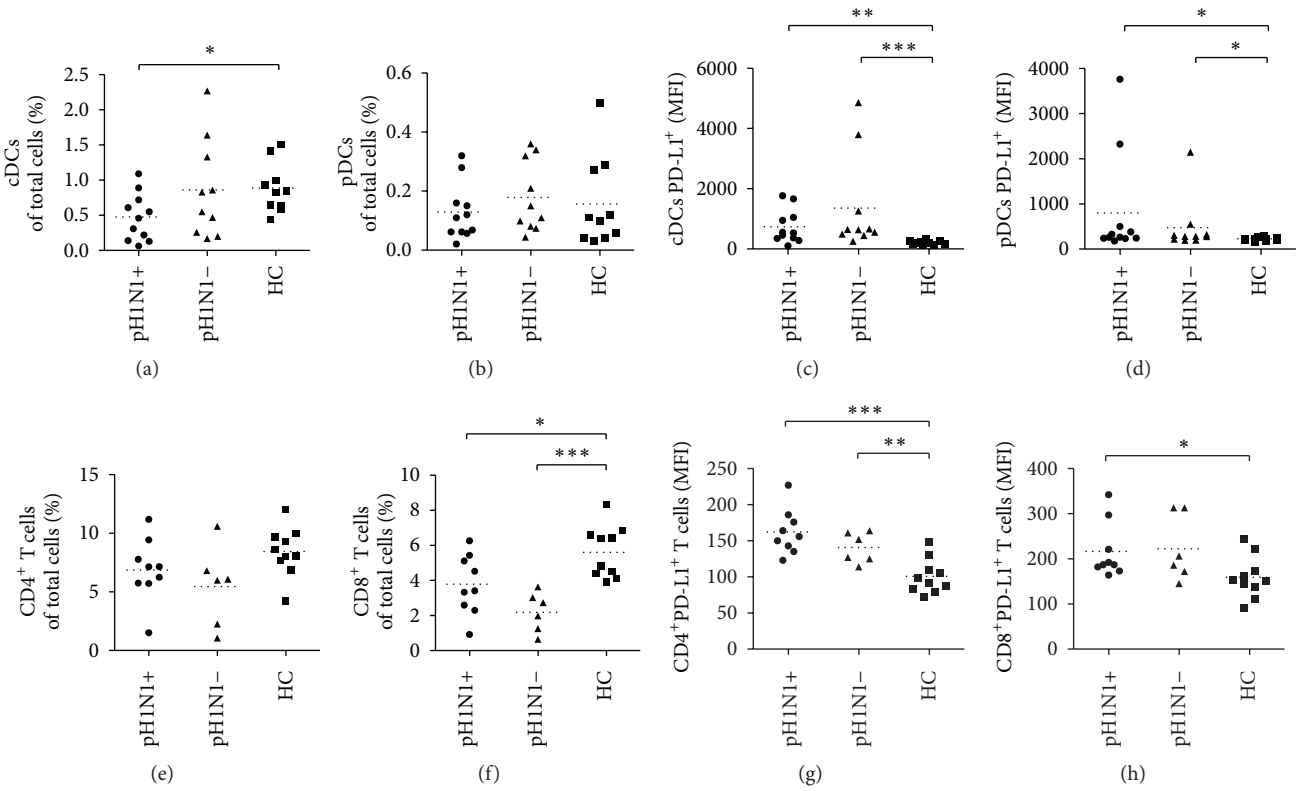

Figure 4: PD-L1 expression is increased on dendritic cells and T cells from PBMCs of patients with acute influenza infection. Cell proportions and surface PD-L1 expression on cDCs and pDCs (a–d) and $CD4^+$ and $CD8^+$ T cells (e–h) from cryopreserved PBMCs from patients with confirmed infection with A(H1N1)pdm09 virus (pH1N1+), patients with influenza-like illness but with a negative RT-PCR result for pandemic H1N1 influenza (pH1N1−), and healthy controls (HC, $n = 10$; error bars indicate SEM) were analyzed by flow cytometry. MFI: mean fluorescence intensity; pH1N1: A(H1N1)pdm09 virus. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (Mann-Whitney test).

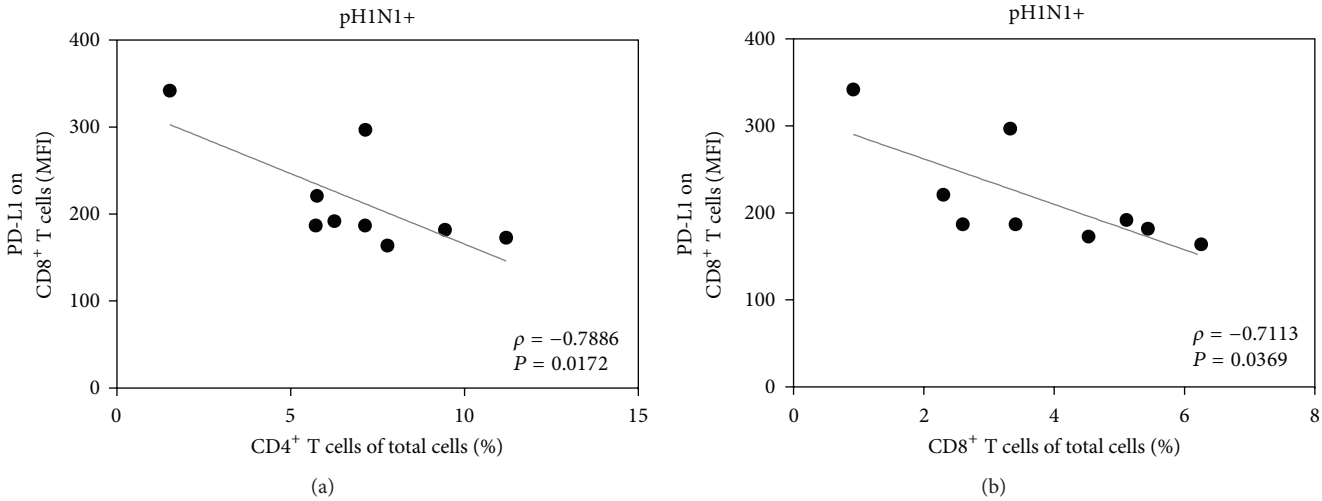

Figure 5: PD-L1 expression on $CD8^+$ T cells is associated with a decreased T cell proportion in patients with acute A(H1N1)pdm09 viral infection. Correlations between PD-L1 expression on the proportions of $CD8^+$ and $CD4^+$ T cells in PBMCs from pH1N1+ patients are shown. Correlations between PD-L1 expression on $CD8^+$ T cells and the proportion of total $CD4^+$ (a) and $CD8^+$ (b) T cells in PBMCs in pH1N1+ patients are indicated. Spearman correlation (ρ) and P values are shown in each graph. MFI: mean fluorescence intensity; pH1N1: A(H1N1)pdm09 virus.

to understanding the regulation of cytokine expression and the control of the exacerbated immune response during infection, as has been previously reported [12].

We observed low frequency of cDCs in the blood of patients infected with A(H1N1)pdm09 during the first and second pandemic waves in Mexico City. It has been reported that in influenza-infected patients, DCs are recruited in the lung, suggesting that the low proportions that we observed may be caused by the redistribution of the DC population from the blood to the lung [30]. Moreover, we observed a decrease in the proportion of $CD8^+$ T cells in influenza-infected patients; thus, $CD8^+$ T cells may also have been redistributed to the lungs. However, we found an increase in PD-L1 expression in DCs and T cells of pH1N1+ patients; it is possible that PD-1/PD-L1 signaling enhanced $CD8^+$ T cell apoptosis as reflected in the decreased T cell proportion and as we showed in the *in vitro* assays results.

Our *in vitro* results showed that unlike the A(H1N1)pdm09 virus, the H3N2 virus did not induce PD-L1 expression either in DCs or T cells; however, in addition to the seasonal H3N2 virus, seasonal H1N1 viruses were also circulating at that time in Mexico, so we cannot rule out that seasonal H1N1 virus could have also induced PD-L1 expression. Considering that our *ex vivo* results showed that PD-L1 up regulation may not be strain specific, and that in the natural infection additional immune mediators may contribute to PD-L1 up regulation, we do not discard the possibility that different types of influenza A virus could induce PD-L1 expression on DCs and T cells during acute infection.

PD-1 and PD-L1 have been recently reported to be expressed in the lungs of A(H1N1)pdm09 patients [10]. Since we did not analyze respiratory tissue samples, it was not possible to determine if the consequences of PD-L1 expression on T cells and DCs that we observed in peripheral blood could reflect the localized response in lungs.

In A(H1N1)pdm09 infected patients, PD-L1 expression on $CD8^+$ T cells is inversely correlated with $CD4^+$ and $CD8^+$ T cell proportions, but this correlation was only observed in pH1N1+ patients; this finding could be explained by the fact that the lymphopenia induced by A(H1N1)pdm09 has been reported to be more severe and refractory than that associated with seasonal infection, which is modest during the first days and resolves earlier [31]. Since we detected PD-L1 expression in both pH1N1+ and pH1N1− patients, we consider that additional factors related to the immune response and inflammation triggered during the acute infection (such as interferons), could be involved in the correlation between PD-L1 expression on $CD8^+$ T cells and the proportion of T cells observed only in pH1N1+ patients [32, 33].

Our data suggest that viral infection may impair the induction of an efficient adaptive immune response in the early stages of infection by promoting PD-L1 expression on DCs and T cells; this could be a mechanism of immune evasion by the A(H1N1)pdm09 virus, similar to that reported in chronic and acute viral infections [6, 27, 34–36]. Since the analyzed patients were recruited at the beginning of the pandemic outbreak in Mexico City, whether these observations are a particular characteristic of early pandemic outbreaks or can also be observed during seasonal outbreaks remains to be elucidated. Our findings suggest that PD-L1 expression could be a useful marker in the evaluation of the early T cell response against influenza infection and may be a possible target for intervention in patients with other acute viral respiratory infections.

5. Conclusion

The 2009 pandemic influenza A(H1N1) virus is able to impair T cell responses through PD-L1 expression, suggesting that the virus could modulate host immune responses during infection by this mechanism.

Abbreviations

PD-L1:	Programmed death ligand-1
PD-1:	Programmed death-1
cDC:	Conventional dendritic cell
pDC:	Plasmacytoid dendritic cell
A(H1N1)pdm09/pH1N1:	2009 pandemic influenza A(H1N1) virus
HC:	Healthy control
PBMC:	Peripheral blood mononuclear cells
SEB:	Staphylococcal enterotoxin B
HA:	Hemagglutinin.

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Research Article

O-Glycosylation of NnTreg Lymphocytes Recognized by the *Amaranthus leucocarpus* Lectin

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O-glycosidically-linked glycans have been involved in development, maturation, homing, and immune regulation in T cells. Previous reports indicate that *Amaranthus leucocarpus* lectin *(ALL)*, specific for glycans containing galactose-N-acetylgalactosamine and N-acetylgalactosamine, recognizes human naïve $CD27^+CD25^+CD4^+$ T cells. Our aim was to evaluate the phenotype of $CD4^+$ T cells recognized by *ALL* in peripheral blood mononuclear cells obtained from healthy volunteers. $CD4^+$ T cells were isolated by negative selection using magnetic beads-labeled monoclonal antibodies; the expression of T regulatory cell phenotypic markers was assessed on *ALL*-recognized cells. In addition, IL-4, IL-10, IFN-γ, and TGF-β intracellular production in ALL^+ cells was also evaluated. The analyses of phenotypic markers and intracellular cytokines were performed through flow cytometry. *ALL*-recognized $CD4^+$ T cells were mainly $CD45RA^+$, $CCR7^+$ cells. Although 52 ± 10% $CD25^+Foxp3^+$ cells were positive to *ALL*, only 34 ± 4% of ALL^+ cells corresponded to $CD25^+Foxp3^-$ cells. Intracellular cytokines in freshly obtained ALL^+CD4^+ T cells exhibited 8% of IL-4, 15% of IL-10, 2% of IFN-γ, and 15% of TGF-β, whereas ALL^-CD4^+ T cells depicted 1% of IL-4, 2% of IL-10, <1% of IFN-γ, and 6% of TGF-β. Our results show that galactose-N-acetylgalactosamine and N-galactosamine-bearing $CD4^+$ T cells expressed phenotypic markers of NnTreg cells.

1. Introduction

Lectins specific for O-glycosidically-linked glycans, containing galactose-N-acetylgalactosamine or N-acetylgalactosamine, have been used to study T-cell activity, as reviewed by [1, 2]. In these studies, the lectin from *Artocarpus heterophyllus* is considered to be mitogenic for human $CD4^+$ T cells [3], whereas *Artocarpus lakoocha* has antiproliferative effect on human leukemic cells [4], and *Dolichos biflorus* agglutinin is used to isolate leukemic T cells [5].

Amaranthus leucocarpus lectin (*ALL*) is a nonmitogenic lectin with specificity for galactose-N-acetylgalactosamine (Galβ1,3 GalNAcα1,O-Ser/Thr) or N-acetylgalactosamine (GalNAcα1,O-Ser/Thr) [6]. In contrast to other GalNAc-specific lectins such as *S. sclarea* [7] or PNA [8], the recognition of *ALL* is limited when GalNAc residues are arranged in clusters, as demonstrated by using glycopeptides with different GalNAc distribution [9, 10]. This lectin binds murine medullary thymocytes [11] and a human peripheral blood $CD4^+$ T cell subset with phenotypic markers CD25, CD27, and CD45RA [12]. Interestingly, *ALL* is capable of inducing suppression of the immune response in mice [13], and it recognizes dexamethasone-resistant thymocytes with increased GalNAc transferase-activity [14]. It has been shown that dexamethasone administration to mice allows the survival of functional $CD4^+CD25^+$ T regulatory cells in

the thymus [15]. Likewise, the dexamethasone treatment in asthma patients promotes differentiation toward T regulatory cells by a Foxp3-dependent mechanism [16], and when patients, receiving allogeneic lymphocyte transplantation, are treated with glucocorticoid, graft versus host disease is suppressed by expansion of $CD4^+CD25^+Foxp3^+$ T cells [17].

The existence of T-cell subsets with regulatory capacity of the immune response, expressing CD4, CD25, and Foxp3 has been evidenced [18]. Naturally occurring $CD4^+CD25^+$ regulatory T cells (nTregs) represent a major lymphocyte population engaged in the maintenance of immune tolerance as reviewed in [18]. $CD4^+CD25^+$ nTregs are differentiated in the normal thymus as a functionally distinct subpopulation of T cells [19, 20]. In humans, the $CD4^+CD25^+$ nTregs are $CD27^+CCR7^+Foxp3^+$, and most of these cells express CD45RO [21]. On the other hand, the existence of $CD45RA^+$ Tregs that resemble a naïve cell subset (NnTreg) have been described [22, 23]. Glycosylation changes have also been reported in nTregs, suggesting that sialylation could be a regulatory ligand in $CD4^+CD25^+$ $Foxp3^+$ cells [24]. In this context, O-glycosylation has been proposed to play a direct and powerful role in regulating T-cell function [14, 25, 26]. Recently, *ALL* has also shown a costimulatory effect on human $CD4^+$ T cell activated via CD3 [27] turning *ALL* into a new tool to study O-glycans-bearing glycoproteins in T-cell populations. Thus, the aim of this work was to know whether the O-glycosidically linked structures recognized by *ALL* are expressed by a Treg subset.

2. Material and Methods

2.1. Antibodies and Reagents. Phycoerythrin (PE)-labeled mouse IgG monoclonal antibodies (mAbs) against human IL-4, IL-10, and CTLA-4 and fluorescein isothiocyanate (FITC)-labeled antibodies against human IFN-γ, CD62L, Foxp3, and CyChrome-streptavidin were purchased from BD PharMingen (San Jose, CA, USA). FITC- and PE-labeled mouse anti-human CD45RA and CD45RO mAbs and anti-IgG-FITC were obtained from Southern Biotech, Inc. (Birmingham, AL, USA). FITC- and PE-labeled mAbs antihuman CCR7 and anti-hLAP (TGF-β1) were from RD Systems (Minneapolis, MN, USA). CD4 T-cell isolation Kit II (MiniMACS system) was obtained from Miltenyi Biotec (Auburn, CA, USA). Lymphoprep (Ficoll 1.077 density) was from Nycomed Pharma (Nyegaard, Oslo, Norway). RPMI-1640 culture medium, concanavalin A, FITC- and PE-labeled streptavidin, N-Acetyl-D-galactosamine (GalNAc), and salts were from Sigma Chemical Co. (St. Louis, MO, USA). Biotin was obtained from Pierce Chem, Co (Rockford, IL, USA). Sodium pyruvate, L-glutamine, and 2-mercaptoethanol were purchased from Gibco BRL. (Rockville, MD, USA). Fetal calf serum was from HyClone Labs. (Logan, UT, USA), and BD Cytofix/Cytoperm Kit and FASTImmune Kit (IFN-γ/IL-4) were from e-Biosciences (San Diego, CA, USA).

2.2. Lectin. Amaranthus leucocarpus seeds were obtained from Tulyehualco, Mexico, and the lectin (*ALL*) was purified by affinity chromatography [6] and subsequently labeled with biotin at a biotin/protein ratio of 2 : 1 [28].

2.3. Isolation of Peripheral Blood Mononuclear Cells (PBMC). Heparinized peripheral blood was diluted 1 : 2 (vol/vol) in phosphate buffered saline (PBS), pH 7.2. PBMC were separated on a Ficoll density gradient by centrifugation at 1700 rpm for 30 min at room temperature (25 ± 3°C). After centrifugation, the interface cells were collected, washed twice, and counted using a hemocytometer to assess viability by trypan blue dye exclusion.

2.4. Isolation of $CD4^+$ T Cells. $CD4^+$ T cells were purified from PBMC by negative magnetic separation in a MiniMACS system. For all cell isolations, the manufacturer's instructions were followed. The purity of the separated $CD4^+$ T cells was determined to be >95% in analyses through a flow cytometry (Becton & Dickinson FACScan; Mountain View, CA, USA), using an antihuman CD4 mAb coupled to FITC. Cell viability (>90%) of the purified cell subset was determined by the trypan blue dye exclusion method.

2.5. Inhibition Assays. Purified $CD4^+$ cells were incubated with optimal concentration of biotin-labeled *ALL* in PBS supplemented with 0.2% bovine serum albumin and 0.2% sodium azide (PBA). After incubation, the cells were washed in PBA and incubated for a second step with PE-labeled streptavidin in PBA. To evaluate specificity of *ALL*, cells were again washed and incubated for the third time with 200 mM GalNAc in PBA. All incubations were carried out during 30 min at 4°C. Cells incubated only with PE-streptavidin were used as staining control.

2.6. Cell Cultures. $CD4^+$ T cells were cultured in 24-well flat bottomed cell culture plates (Costar, Cambridge, MA, USA) at 1×10^6 cells/well in RPMI-1640 medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/mL gentamicin, and 10% heat-inactivated fetal calf serum and incubated at 37°C in a 5% CO_2 humidified chamber. Concanavalin A (2 μg/mL) was added and, after 48 h, the cells were harvested and processed to measure Foxp3, TGF-β, and CTLA-4 expression through flow cytometry.

2.7. Immunofluorescence Staining of Cell Surface Markers. Triple-color staining was performed on purified $CD4^+$ T cells by direct immunofluorescence, using FITC- or PE-labeled mAbs against CD45RA, CD45RO, CCR7, or CD25, and indirect fluorescence, using an optimal concentration of biotin-*ALL* plus CyChrome-labeled streptavidin. Briefly, 2×10^5 cells were suspended in 20 μL PBA and incubated with the fluorochrome-labeled mAb and biotin-*ALL* for 30 min at 4°C. After incubation, the cells were washed twice with PBA and incubated with CyChrome-streptavidin for 30 min. Then, cells were washed twice with PBA, fixed with 1% *p*-formaldehyde, and analyzed by flow cytometry.

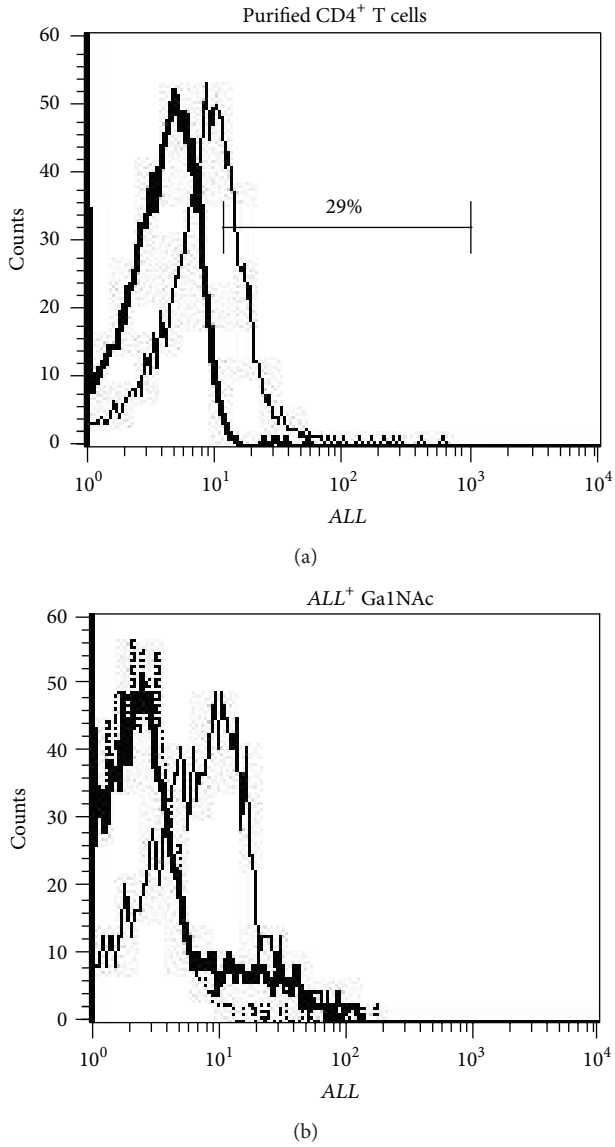

Figure 1: *ALL* recognizes purified $CD4^+$ T cells. (a) Freshly purified $CD4^+$ T cells were stained with CyChrome-labeled streptavidin alone (thick line) after incubation with biotin-labeled *ALL* (thin line). The bar denotes percentage of purified ALL^+CD4^+ T cells. (b) GalNAc inhibited *Amaranthus leucocarpus* lectin (*ALL*) binding sites for $CD4^+$T cells. Gray lines denote the staining of cells treated only with CyChrome-streptavidin; thin line indicates the florescence level of ALL^+ cells; thick line denotes fluorescence of ALL^+ cells when they were treated with GalNAc.

2.8. Flow Cytometric Analysis of Intracellular Proteins. Purified $CD4^+$ cells were incubated with biotin-*ALL* and CyChrome-streptavidin, as described above. Then, cells were fixed and permeabilized with the BD Cytofix/Cytoperm kit. Individually, cells were incubated with mAbs anti-IFN-γ FITC/IL-4 PE, anti-IL-10 PE, anti-Foxp3-FITC, or anti-CTLA-4-PE or anti-hLAP (TGF-β). After 30 min, cells were washed with PBS, incubated with anti-IgG-FITC for 30 min, and finally analyzed by flow cytometry.

Table 1: Recognition of $CD25^+Foxp3^+CD4^+$ T-cell subsets by *Amaranthus leucocarpus* lectin (*ALL*).

$CD4^+$T cells			
$CD25^+Foxp3^-$ cells		$CD25^+Foxp3^+$ cells	
ALL^+	ALL^-	ALL^+	ALL^-
$34 \pm 4^*$	$65 \pm 4^{*\ddagger}$	52 ± 10	$50 \pm 7^{\ddagger}$

$^*P = 0.001$, $^{\ddagger}P = 0.004$.

2.9. Flow Cytometric Analysis. All cells were analyzed for marker's expression by collecting 5000 events using a FACScan flow cytometer (Becton Dickinson, Mountain, View, CA, USA) and Cell Quest Pro software. To evaluate cell surface marker staining, a gate was drawn around the lymphocyte populations based on their physical properties (forward and side scatter), and a second gate was drawn based on positive or negative fluorescent *ALL*-binding to $CD4^+$ T cells. To analyze intracellular protein staining, positive fluorescence staining of IFN-γ, IL-4, IL-10, TGF-β, Foxp3, and CTLA-4 (forward scatter and fluorescence) was set manually based on the distribution of cells stained with the isotype controls. Data are presented as two-dimensional dot plots or histograms. Control stains were performed using isotype-matched mAbs of unrelated specificity that were labeled with FITC or PE. Background staining was <1% and was subtracted from experimental values.

2.10. Statistical Analysis. Experiments were done independently and repeated at least three times. Data were analyzed by Mann-Whitney *U* test to detect significant differences. Analyses were performed with Sigma-Stat 3.1 software. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Flow Cytometric Phenotypic Analysis. The ALL^+ T cell subset corresponded to 29% of the purified $CD4^+$ T cell population (Figure 1(a)). The specific interaction of *ALL* with a subset of $CD4^+$ T cells was confirmed by inhibition assays with their characteristic ligand, as expected GalNAc inhibited most of *ALL*-binding sites for $CD4^+$ cells (Figure 1(b)). Phenotypic characterization of both ALL^+ and ALL^-CD4^+ T cells showed that the frequency of $CD62L^+$ cells was 1.1 times higher in ALL^+CD4^+ T cells (78% ± 1.4) than in ALL^-CD4^+ T cells (69.2% ± 2.4) ($P = 0.03$). In ALL^+CD4^+ T cells, the percentage of $CD45RA^+$ cells was higher (71%) than the percentage of $CD45RO^+$ cells (9%) ($P = 0.02$) (Figure 2(a)). We observed also that the frequency of $CCR7^+$ cells was increased 1.5 times more in ALL^+CD4^+ T cells (72%) than in ALL^-CD4^+ T cells (41%) (Figure 2(b)).

3.2. Frequency of CD25 and Foxp3 on $CD4^+$ T Cells. Despite that the frequency of $CD25^+Foxp3^+$ cells was similar in both ALL^+ and ALL^- T cells (Table 1), we observed that $CD25^+Foxp3^-$ cells were 1.9 times more frequent in ALL^- T

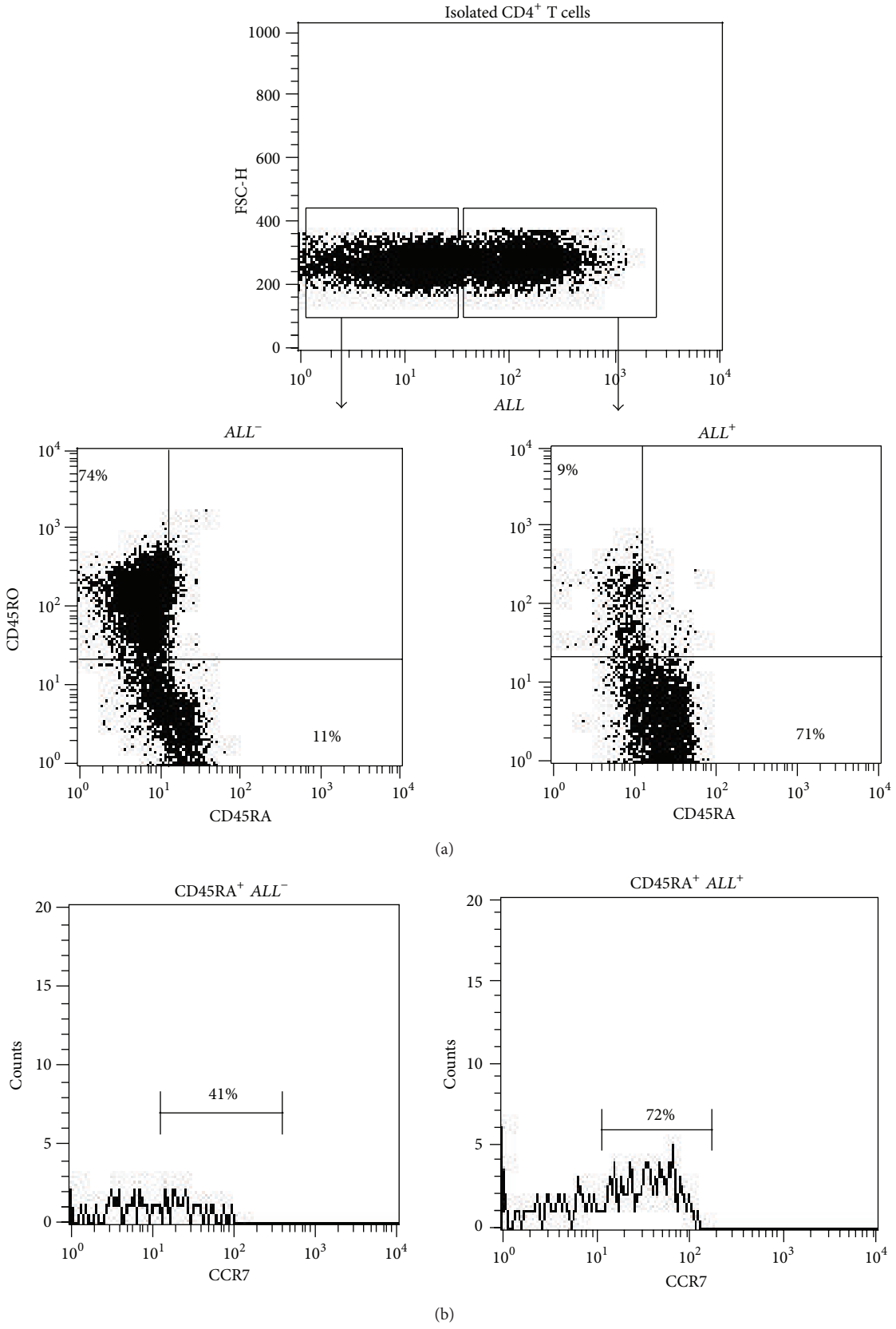

Figure 2: *ALL* recognition of $CD4^+$ T cells with phenotype positive to CD45RA and CCR7 markers. (a) Dot plots of ALL^- and ALL^+ gated cells showing frequency of $CD45RO^+$ and $CD45RA^+$ cells. (b) Representative histograms of $CD45RA^+$ gated cells to analyze CCR7 frequency in both ALL^- and ALL^+ T helper cells. The numbers denote percentage of cells positive to the marker.

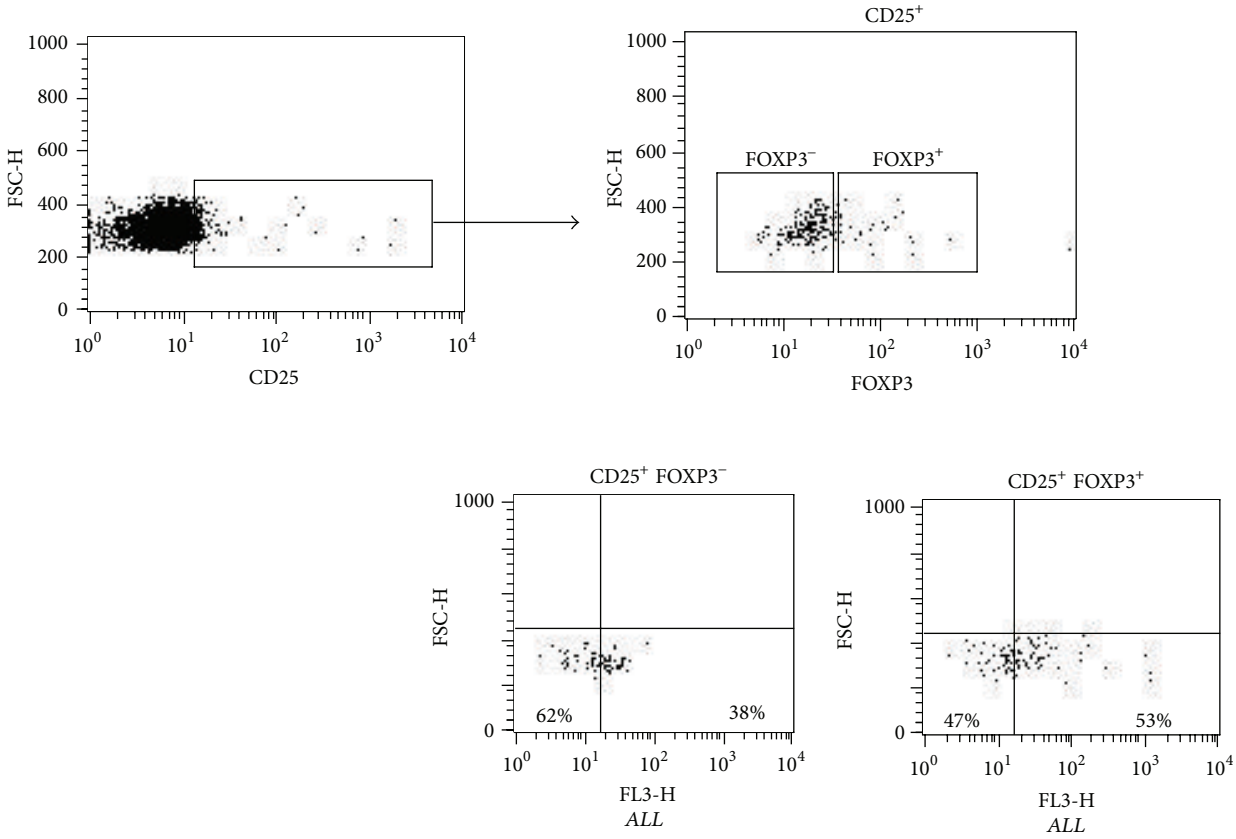

Figure 3: CD25 and Foxp3 frequency on ALL^- and ALL^+CD4^+ T cells. To analyze cell surface marker staining, a gate was drawn around the lymphocyte population based on their physical characteristics (forward) and positive fluorescence to CD25; a second gate was drawn based on positive or negative fluorescence to Foxp3. $CD25^+$ cells positive or negative to Foxp3 were analyzed by binding to *ALL*.

Table 2: Frequency of TGF-β^+ cells on purified $CD4^+$ cells.

Con A-stimulated	Nonstimulated
ALL^+ cells 94 ± 6*	4 ± 2*
ALL^- cells 38 ± 18	6.7 ± 5.7

*$P = 0.003$.

helper populations (62%) than in ALL^+ T helper cells (38%) ($P = 0.001$). Similarly, $CD25^+Foxp3^+$ cells were 1.3 times less frequent in ALL^-CD4^+ T cells (47%) ($P = 0.04$) than in ALL^+CD4^+ T cells (53%) (Figure 3).

3.3. Intracellular Cytokines in ALL^+CD4^+ T Cells. To know the cytokine profile of ALL^+ and ALL^- T helper cells at basal conditions, we performed intracellular staining to the cytokines IL-4, IL-10, IFN-γ, and TGF-β in nonstimulated $CD4^+$ cells. Our results showed that ALL^+CD4^+ cells were IL-10^+ (15%), IL-4^+ (8%), IFN-γ^+ (2%), and TGF-β^+ (15%); in contrast, the percentages of cells positive to intracellular cytokines in the ALL^-CD4^+ T cells subset were as follows: IL-10^+ cells (2%), IL-4^+ cells (1%), IFN-γ^+ cells (<1%), and TGF-β^+ cells (6%) (Figure 4).

3.4. Frequency of CD25, Foxp3, CTLA-4, and TGF-β Cells after Polyclonal Stimulation. To determine whether the polyclonal stimulation influenced the frequency of CD25, Foxp3, and TGF-β in ALL^+ and ALL^- cells, we performed an *in vitro* Con A stimulation assay during 48 hours. We observed that the frequency of $ALL^-CD25^+Foxp3^+CD4^+$ T cells was increased 66.7 times in Con A-stimulated cells when they were compared to nonstimulated cells ($P = 0.007$), whereas the frequency of $ALL^+CD25^+Foxp3^+CD4^+$ T cells was increased 2-times in ConA-stimulated cells as compared to nonstimulated cells ($P = 0.036$) (Figure 5(a)). Despite the increment in the number of $CD25^+Foxp3^+CD4^+$ T cells in the ALL^- cell subset, the percentage of $CD25^+Foxp3^+CD4^+$ predominated in the ALL^+ cell subset ($P = 0.007$) at the end of the culture (Figure 5(b)). Although, we did not find statistical differences in intracellular/surface frequency of CTLA-4^+ cells among ALL^+ (37.5 ± 6.5) and ALL^- (15.1 ± 2) T helper cells ($P = 0.05$) (data not shown); the percentage of TGF-β^+ cells was statistically higher in ALL^+CD4^+ T cells (98%) than in ALL^-CD4^+ T cells (51%) ($P = 0.02$) (Figure 5(c)). After polyclonal stimulation, the percentage of TGF-β^+ T cells increased 2.6 times in the ALL^+CD4^+ T cell subset as compared to ALL^-CD4^+ T cells; no significant differences were observed when we compared

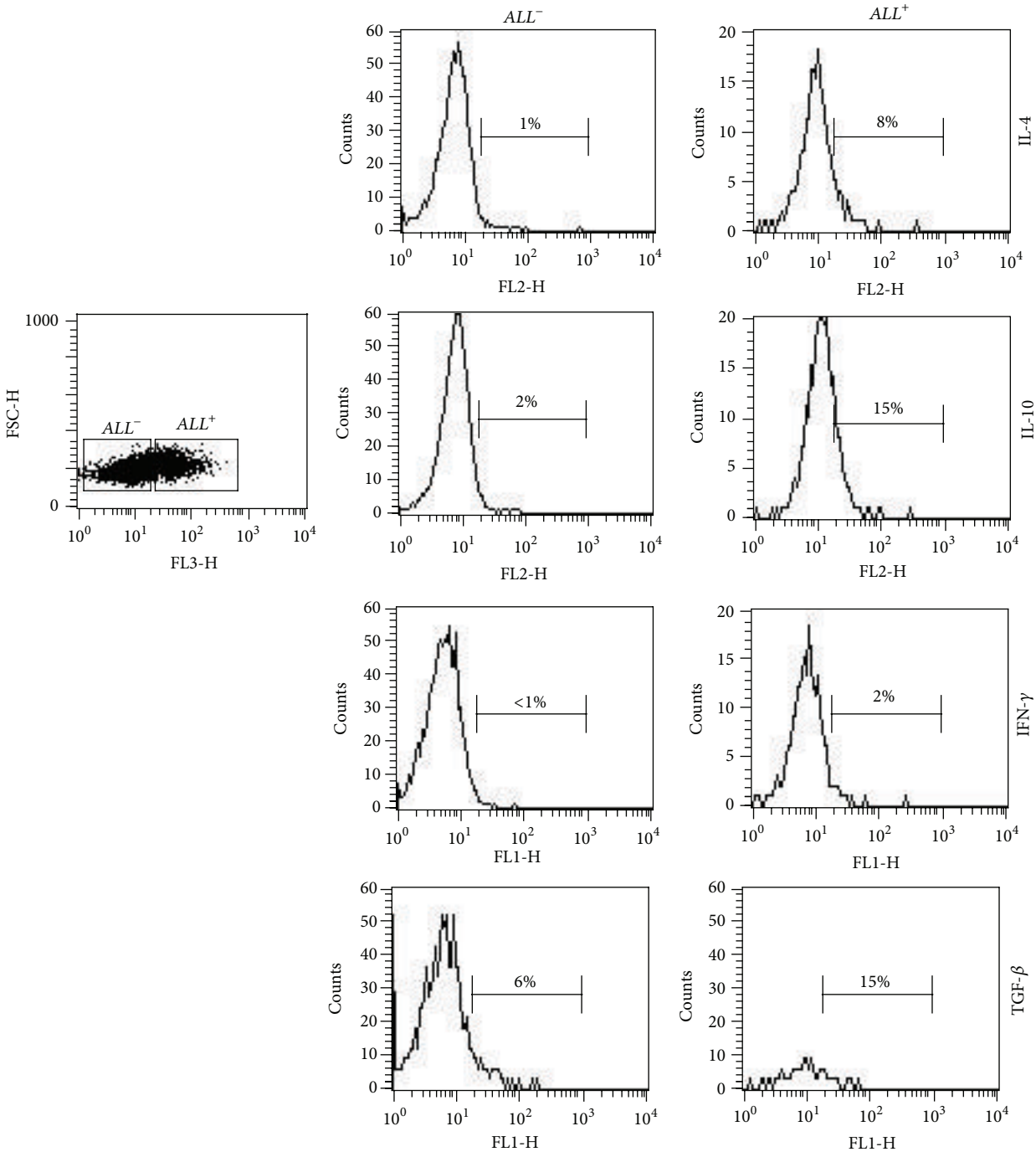

Figure 4: Frequency of intracellular cytokine positive cells on ALL^- and ALL^+ gated cells. Freshly purified $CD4^+$ T cells were incubated with *ALL*-Biotin followed by CyChrome-streptavidin incubation and stained with monoclonal antibodies against IL-4, IL-10, IFN-γ, or TGF-β. Cells were analyzed by flow cytometry and gated on ALL^- or ALL^+ cells. Percentages of cells positive to intracellular cytokines are shown in histograms.

Con A-stimulated ALL^-CD4^+ T cells with nonstimulated cells (Table 2).

4. Discussion

Surface O-glycosylation pattern of lymphocytes has been involved in development, maturation, homing, and immune regulation [24, 25, 29]. It has been shown that glycosylation changes occur in activated lymphocytes [26], and differences in sialylation as well as in expression of O-glycans are related to control of T-cell activation [30]. In this study, we found that a subpopulation of $CD25^+Foxp3^+CD4^+$ T cells expressing galactose-N-acetylgalactosamine or N-acetylgalactosamine is recognized by the *Amaranthus leucocarpus* lectin. A significant percentage of freshly obtained *ALL*-recognized T cells exhibited intracellular cytokines with regulatory activity such

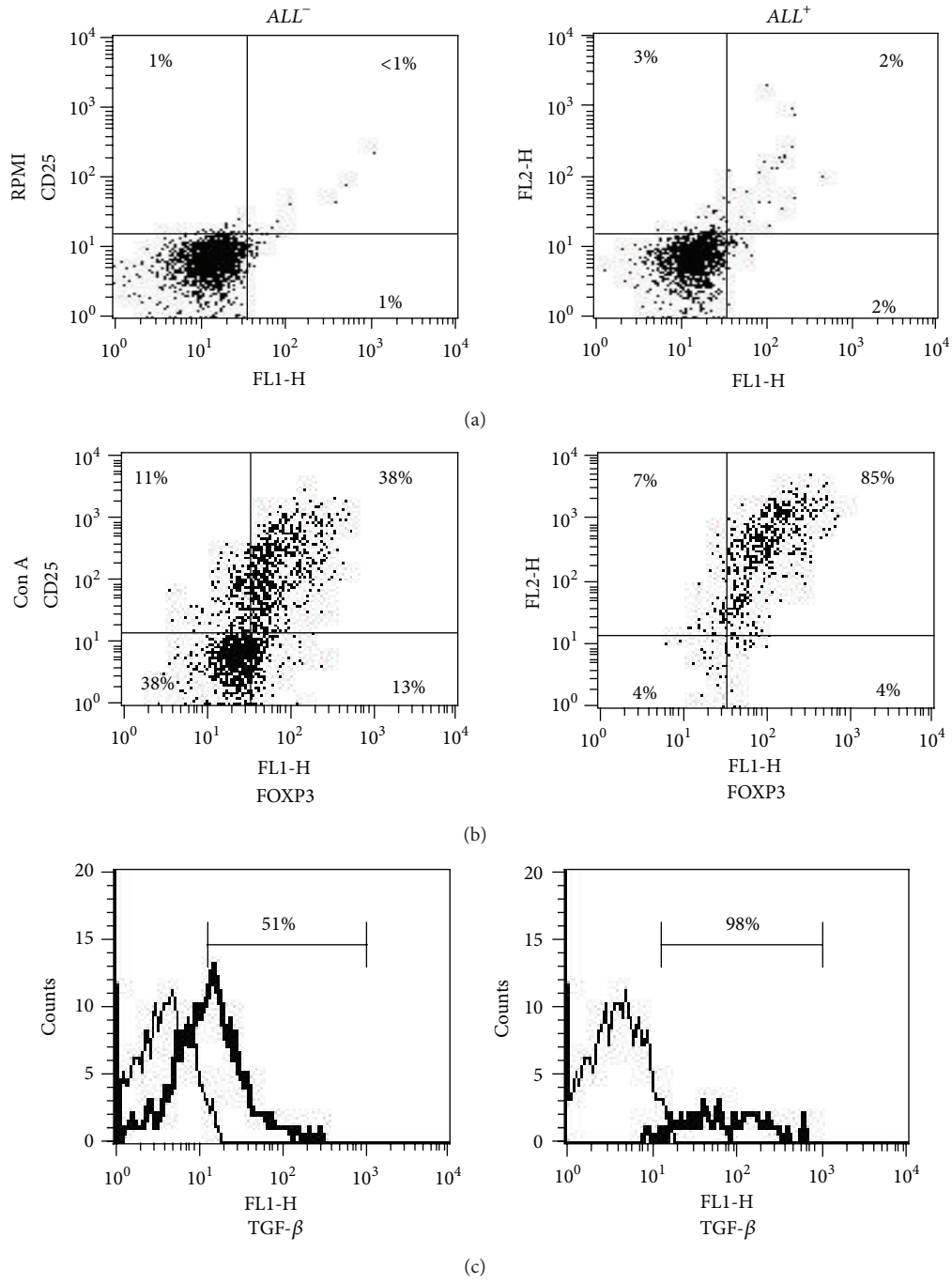

Figure 5: Polyclonal stimulation induced high expression of Foxp3 and TGF-β in ALL^+ T helper cells. $CD4^+$ T cells were stimulated with Concanavalin-A (2 μg/mL). After 48 h, cells were stained with biotin-*ALL* followed by CyChrome-streptavidin, and stained with monoclonal antibodies against CD25 and Foxp3 or TGF-β. (a) Dot plots of nonstimulated cells (RPMI alone) showing frequency of $CD25^+Foxp3^+$ in ALL^- and ALL^+ T helper cells. After polyclonal stimulation An increased frequency of both (b) $CD25^+Foxp3^+$ cells and (c) TGF-β^+ cells (thick line) in ALL^+ T helper cells was observed. (Thin line corresponds to isotype control).

as IL-10 and TGF-β. It has been shown that $CD25^+CD4^+$ Treg cells are able to produce IL-4 and IL-10, without production of IFN-γ [23]. Likewise, other authors have shown that sorted $CD25^+CD4^+$ Treg cells produce high concentrations of IL-10 and low IFN-γ [22]. In agreement with these data, our results suggest that ALL^+ T cells correspond to a Treg cell subset.

Previous reports concerning *ALL* binding to T cells suggest that this lectin recognizes specific O-glycans on recently activated naïve T cells [31]. Our results showed that ~29% of the purified $CD4^+$ T cells were positive to *ALL*. Phenotypic characterization of ALL^+CD4^+ T cells included a subset of $CD45RA^+$, $CCR7^+$, $CD25^+$, and $Foxp3^+$ cells, which resemble natural naïve NnTregs [32]. The NnTregs

represent a major lymphocyte population engaged in the control of self-reactive T responses and in maintenance of immune tolerance [18, 20]. Along with our findings, $CD4^+$ T cells recognized by *ALL* were reported $CD45RA^+CD27^+$ cells [12]. The CD27 molecule is a member of the TNF receptors that have been involved in early activation of naïve cells [33]. It is interesting to note that CD27 expression on $CD4^+$ T cell lines discriminates between regulatory and nonregulatory cells [33, 34]. Different authors have reported a distinct subset of $CD25^+Foxp3^+CD4^+$ T cells characterized by CCR7, CD62L, and CTLA-4 expression contained in the $CD45RA^+/RO^-$ naïve compartment [22, 23, 35]. CCR7 is a chemokine receptor that controls homing of lymphocytes to secondary lymphoid organs, and its expression in Tregs has been associated with maintenance of these cells for prolonged periods of time at those sites, inhibiting effector T-cell expansion [21]. CD62L and CCR7 expression in T cells mediates lymphocyte homing to secondary lymphoid organs [36]. CTLA-4 is a cell surface molecule that is expressed rapidly before cell activation, surface CTLA-4 is immediately internalized, which could explain the low levels of expression generally detected on the cell surface [37]. Diverse authors have related CTLA-4 expression with nTreg cells [22, 23], and CTLA-4 expression is increased in Treg > NnTreg. Consistent with these data, we observed that the percentage of $CTLA\text{-}4^+$ cells was increased in ALL^+ than in ALL^-CD4^+ T cells, suggesting that $CD4^+$ cells recognized by *ALL* could be either a subpopulation enriched in NnTreg cells or recently activated Treg cells [23]. A proposal could be explained based on results obtained with *in vivo* antigenic-experienced circulating cells, as has been reported for other proteins related to Treg cells, such as Foxp3 [19], or for the intracellular CTLA-4 [22, 23].

Regulatory T cells are resistant to apoptosis [38]; similarly, ALL^+ thymocytes are resistant to apoptosis after treatment with dexamethasone [14]; for this reason it would be interesting to examine apoptosis resistance in the ALL^+CD4^+ T cells from human peripheral blood to understand better the potential regulatory characteristics of this ALL^+ NnTreg cell subset. Golks et al. [26] showed that an N-acetylglucosaminyltransferase is required for T cell activation, suggesting that modifications in glycosylation accompany T cell activation as reviewed [29]. In this regard, the glycosylation status could generate differences among subsets of T cells, and possibly Tregs, as we observed in this study.

Taken together, our results suggest that *ALL* constitutes an important tool to study differences in O-glycans on $CD4^+$ T cells with a regulatory-like phenotype, these $CD4^+$ T cells are enriched NnTregs or recently activated Tregs expressing galactose-N-acetylgalactosamine and N-acetylgalactosamine.

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Research Article

Low-Dose Amphotericin B and Murine Dialyzable Spleen Extracts Protect against Systemic *Candida* Infection in Mice

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Candida albicans causes opportunistic systemic infections with high mortality (30%–50%). Despite significant nephrotoxicity, amphotericin (AmB) is still used for the treatment of this serious fungal infection. Therefore, alternative treatments are urgently needed. Dialyzable leukocyte extracts have been used successfully to treat patients with mucocutaneous candidiasis, but their effectiveness in systemic candidiasis has not been evaluated. In this study, low-dose AmB (0.1 mg/kg) plus 10 pg of murine dialyzable spleen extracts (mDSE) were tested in a systemic candidiasis mouse model. Survival, tissue fungal burden, kidney damage, kidney cytokines, and serum levels of IL-6 and hepcidin were evaluated. Our results showed that the combined treatment of low-dose AmB plus mDSE improved survival and reduced kidney fungal burden and histopathology; these effects correlated with increased kidney concentration of IFN-γ and TGF-β1, decreased levels of TNF-α, IL-6, and IL-10, as well as high levels of systemic IL-6 and hepcidin. Low-dose AmB and mDSE synergized to clear the infectious agent and reduced tissue damage, confirming the efficacy of a low dose of AmB, which might decrease the risk of drug toxicity. Further studies are necessary to explore these findings and its implications in future therapeutic approaches.

1. Introduction

Opportunistic mycoses are infections caused by saprophytic or commensal fungi in hosts in which the normal microbiota has been altered by antibiotic treatments, in hosts with disrupted anatomic barriers, or in immunosuppressed hosts [1]. *Candida albicans* is an ubiquitous, dimorphic fungus that colonizes the skin, gastrointestinal tract, and oral and vaginal mucosa of immunocompetent individuals without causing disease [2], but it can cause opportunistic mucocutaneous and systemic infections (with a mortality of 30–50%), particularly in hospitalized patients [1, 3, 4].

Neutrophils and macrophages play a central role in the immune response against *C. albicans*, and decreased

numbers of these cells correlate with increased tissue fungal burden and mortality [5]. Neutrophils and macrophages are activated through several pattern-recognition receptors, including Toll-like receptor (TLR) 2, TLR4, TLR9, C-type lectin receptor, dectin-1, dectin-2, DC-SIGN, mincle, galectin-3, SCARF1/CD36, and NLRP3. Recognition of *C. albicans* by dectin-1 triggers CARD9 signalling, and mutations in CARD9 lead to chronic mucocutaneous candidiasis and invasive *Candida* infections in humans [6]. The proinflammatory cytokines TNF-α and IL-6 are also critical for the immune response against *C. albicans* [5].

The recognition of *C. albicans* yeasts by TLR4 leads to the production of IL-12 and a Th1 response [7], while the recognition of hyphae by dectin-1 and dectin-2 triggers the production of IL-23 and a Th17 response [7]. The recognition of hyphae by TLR2 is associated with the induction of a Th2 response [7], which is not protective but could be involved in the regulation of the inflammatory response, since mice that lack TLR2 are resistant to systemic candidiasis [8, 9].

Several antifungal drugs are available for the treatment of *C. albicans* infections, including amphotericin B (AmB), 5-fluorocytosine, fluconazole, itraconazole, voriconazole, posaconazole, and ravuconazole, but their use is limited because of their toxicity and their low efficacy rates [10]. Echinocandins are a new class of antifungal drugs that inhibit the synthesis of β-glucan in *C. albicans* cell wall. They are effective against most isolates of *Candida* spp. and they are less toxic than other antifungal drugs, but they are expensive [11]. Dialyzable leukocytes extracts (DLE) are low molecular weight-dialyzable peptides from immune cells, which have immunomodulatory activities [12]. DLE have been used in clinical settings for the treatment of several diseases, including herpes zoster, herpes simplex type I, herpetic keratitis, atopic dermatitis, osteosarcoma, tuberculosis, asthma, postherpetic neuritis, anergic coccidioidomycosis, leishmaniasis, toxoplasmosis, sinusitis, pharyngitis, and otitis media [13]. Intramuscular DLE have been used successfully to treat mucocutaneous candidiasis in humans [13–17].

In a previous report, we showed that experimental murine tuberculosis could be successfully treated with a combination of murine dialyzable spleen extracts (mDSE) and conventional chemotherapy [18]. Here, we established an animal model of systemic candidiasis, where the efficacy of low-dose AmB supplemented with mDSE could be assessed. We evaluated the effects of the combined treatment on survival, tissue fungal burden, tissue damage, kidney cytokines, and hepcidin and IL-6 serum levels. We provide evidence that the combination of low-dose AmB plus mDSE is effective for the control of murine systemic candidiasis.

2. Materials and Methods

2.1. Ethics Statement. This study was carried out in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals (NOM-062-ZOO-1999) of the "National Technical Consultation Council for Animal Wellbeing" (CONASA), Ministry of Health, Mexican Government. The protocol was approved by the "Investigation Committee for the Transference Factor Project (CIPFT)" of the National School of Biological Sciences, IPN (Authorization no. IB-10-004).

The followup of all the experimental groups was documented daily by trained animal caretakers. In the experiments where survival was being evaluated, mice were separated from their experimental group and humanely sacrificed by cervical dislocation, when signs of distress (significant weight lost, fever, piloerection, and hyperventilation) were detected. When organs were collected, the mice were previously euthanized by cervical dislocation.

2.2. Experimental Model of Systemic Candidiasis. *C. albicans* 07-387 (*Ca*07-387) was isolated from a patient with systemic candidiasis at the UANL. *Ca*07-387 was cultured at 37°C for 18 h on a rotating drum in Sabouraud medium (Difco, Sparks, MD, USA) and frozen at 5×10^6 CFU/mL in 30% glycerol. For each experiment, a vial was thawed and yeasts were cultured to exponential phase.

To establish the best infecting dose, groups of 5 to 10 female BALB/c mice (4-5 weeks old, 14–16 g) were infected intravenously (i.v.) with different amounts of *Ca*07-387 blastospores in 0.1 mL of sterile saline solution and observed for 30 days. (older mice, 8–12 weeks old, were resistant to the infection). The 5×10^5 dose was chosen for the rest of experiments. Groups of 5 infected mice were treated with different concentrations of AmB (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 mL of water (i.v.), on days 2, 3, 4, 5, and 6 after infection.

2.3. Preparation of Murine Dialyzable Spleen Extracts (mDSE) and Treatment of Infected Mice. mDSE was obtained from 10 healthy adult BALB/c mice (10–12 weeks old). Spleen cell suspensions were disrupted by five cycles of freezing and thawing (−20°C/37°C). Lysates were subjected to three cycles of filtration (2,300 g for 15 min) using Centricon centrifugal filter devices (Millipore, Billerica, MA, USA), with a nominal molecular weight limit of 10 KDa. Filtrates (mDSE) were tested for endotoxin (Gel clot LAL method, Charles River Endosafe, Charleston, SC, USA), sterility, and total peptide content (bicinchoninic acid assay, Pierce Biotechnology, Rockford, IL, USA). The mDSE preparation was sterile and had <0.125 endotoxin units/mL and 96 μg peptides/mL. Infected mice were injected intramuscularly (i.m.) with 10 pg of mDSE, alone or in combination with 0.1 mg/kg AmB (i.v.), on days 2, 3, 4, 5, and 6 after infection.

*Ca*07-387 infected mice were divided into four experimental groups (each with 20–25 mice). Each group received a different treatment: 0.1 mg/kg AmB, 10 pg mDSE, 0.1 mg/kg AmB, and 10 pg mDSE, or saline. Three mice from each group were euthanized on days 2, 10, 15, and 30 after infection. Kidneys, spleens, livers, and brains were weighed, macerated, diluted with saline, and cultured overnight on Sabouraud dextrose agar (Difco) to determine tissue fungal burden. Blood samples were taken from these mice by facial vein puncture, in accordance with the Official Mexican Guidelines (NOM-062-ZOO-1999), and serum aliquots were frozen at −20°C.

2.4. Histopathological Analysis of Tissue Samples. The kidneys, spleen, liver, and brain of mice were taken at the indicated time points and immediately fixed by immersion in 10% formaldehyde/PBS, dehydrated in ethylic alcohol, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (HE), or Gomori-Grocott methenamine silver nitrate staining method (GG). Slides were analyzed under light microscopy (Olympus BX40).

2.5. Hepcidin and Cytokine Quantification. Hepcidin was quantified in serum samples by ELISA. The assay was set using mouse hepcidin (HEPC11-P, Alpha Diagnostic International, San Antonio, TX, USA), rabbit anti-mouse hepcidin antibody (HEPC11-A, Alpha Diagnostic International), and a protein A-HRP conjugate (Bio-Rad, Hercules, CA, USA). IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, and IL-17A were quantified in macerated kidneys and serum samples using Cytometric Bead Array multiplexed bead-based immunoassays (BD Biosciences, San Jose, CA, USA); 2,500 events were acquired for each sample in a FACSAria flow cytometer (BD). Data were analysed using FlowJo software (TreeStar, San Carlos, CA, USA). TGF-β1 was quantified with an ELISA kit (e-Biosciences, San Diego, CA, USA).

2.6. Statistics. Survival curves were analyzed with Kaplan-Meier log-rank test, and CFU and cytokines were analyzed with two-way ANOVA and Bonferroni posttest.

3. Results

3.1. Treatment with Low-Dose AmB Plus mDSE Increases Survival in Mice with Systemic Candidiasis. All mice infected i.v. with 5×10^6, 1×10^6, and 5×10^5 CFU presented piloerection, fever, and significant weight loss (data not shown) and died after 1, 5 and 11 days, respectively. In contrast, mice infected with 2×10^5 and 1×10^5 CFU showed 40% and 60% of survival after 30 days, respectively (Figure 1(a)). The surviving animals did not show any signs of infection at this time. We used 5×10^5 CFU for all further experiments, because this dose provided sufficient time to test the effect of different treatments. The administration of 0.1 mg/kg of AmB (low-dose AmB) to infected mice did not prevent their death but extended their lifespan to 28 days (Figure 1(b)). For this reason, we chose this dose to evaluate the effect of mDSE. A high dose of AmB (2 mg/kg) was required to prevent death of all infected animals (Figure 1(b)).

The administration of 10 pg of mDSE alone did not affect the survival of mice infected with 5×10^5 CFU. In contrast, the administration of low-dose AmB in combination with 10 pg of mDSE produced 100% survival (Figure 1(c)). Mice treated with low-dose AmB plus mDSE showed a significant decrease in kidney fungal burden since day 10 after infection, when compared to mice treated only with mDSE or low-dose AmB (Figure 1(d)). The AmB group controlled kidney fungal burden until day 8 (2 days after the last administration of AmB), when the fungi started to grow exponentially. Fungal burdens in the spleens, livers and brains were 2-log lower than in the kidneys, and no differences in the fungal burden of these organs were observed between the groups (data not shown).

3.2. Treatment with AmB Plus mDSE Ameliorates the Histopathology Induced by Systemic Candidiasis. Mice infected with *Ca*07-387 showed progressive kidney damage: after 2 days of infection, well-defined abscesses with abundant neutrophils (arrows, Figure 2(a)) and yeasts (arrow, Figure 2(f)) were observed in the cortical and medullar regions. These abscesses were larger after 10 days of infection; numerous proximal convoluted tubules had necrotic and detached epithelial cells (arrow, Figure 2(b)), some tubules were completely denuded (asterisks, Figure 2(b)), and abundant hyphae were present (Figure 2(g)).

The histopathological changes in mice infected with *Ca*07-387 and treated with mDSE were similar to those of untreated mice; their kidneys showed necrosis in the pelvic area (Figure 2(c)) and abundant hyphae (Figure 2(h)). The kidneys from mice infected with *Ca*07-387 and treated with AmB showed considerable fibrotic scars (arrow, Figure 2(d)), abundant yeast in the tubular regions (arrows, Figure 2(i)), and no evidence of tubular damage. Interestingly, the kidneys from mice infected with *Ca*07-387 and treated with mDSE and AmB showed limited scar tissue (arrow, Figure 2(e)), no signs of tubular damage, mild inflammation, and few yeast (arrows, Figure 2(j)). No significant histological differences were observed in spleens, livers, and brains from these four experimental groups.

3.3. Effects of Low-Dose AmB and mDSE on Kidney Cytokines, Systemic IL-6, and Hepcidin on Mice with Systemic Candidiasis. The combined treatment modulated the levels of kidney cytokines in mice with systemic candidiasis on day 4 after infection (2 days after treatment initiation). IFN-γ and TGF-β1 concentrations were significantly increased (Figures 3(a) and 3(b)), while IL-6, IL-10, and TNF-α were decreased in comparison with the AmB group (Figures 3(c), 3(d), and 3(e)). No differences were found in IL-2, IL-4, or IL-17A levels (data not shown). Serum IL-6 concentration was significantly higher ($P < 0.001$) on day 10 after infection in mice that received the combined treatment, when compared with mice treated with AmB alone (Figure 3(f)). No differences were detected for serum IFN-γ, TGF-β1, TNF-α, IL-2, IL-4, IL-10, or IL-17A (data not shown).

Serum hepcidin was significantly higher on day 8 in mice that received the combined treatment, compared with mice that were treated with low-dose AmB alone; in the latter group, serum hepcidin increased its concentration only after day 15 (Figure 3(g)).

4. Discussion

The intravenous infection model of systemic candidiasis recapitulates several features of the human disease [5]. In this model, fungal cells are delivered directly to the bloodstream, and infection is controlled in most organs (including the liver and the spleen), but not in the kidneys and (in cases of high inoculum levels), the brain. Mice die of progressive sepsis and develop renal failure, whose severity correlates

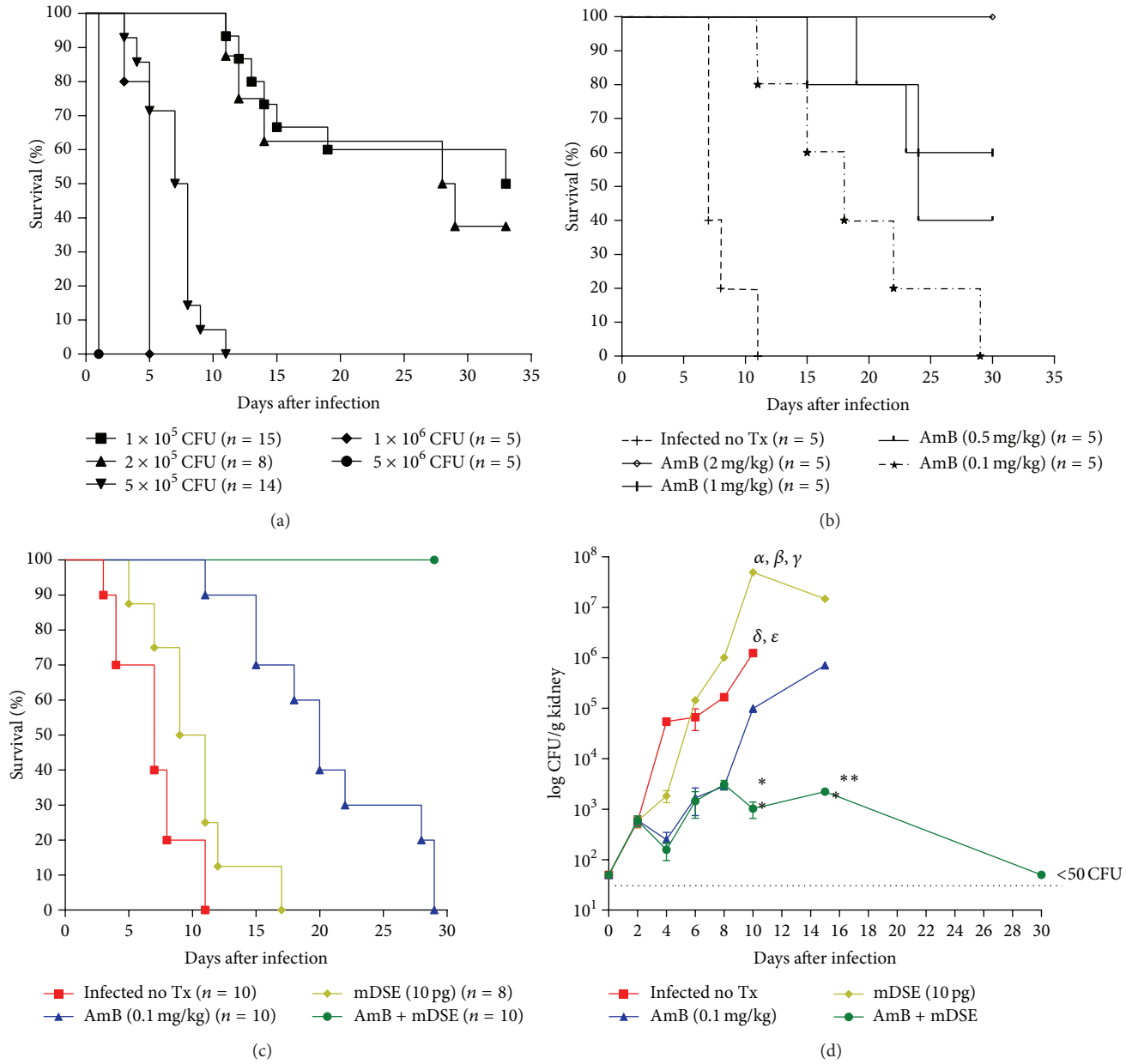

Figure 1: Effect of low-dose AmB and mDSE on murine systemic candidiasis. (a) Mice were infected with the indicated amounts of *Ca*07-387 blastospores and were observed for 30 days. Data from two independent experiments were used. (b) Different concentrations of AmB were administered to mice that had received 5×10^5 CFU of *Ca*07-387. (c) Mice that had received 5×10^5 CFU of *Ca*07-387 were treated with 10 pg of mDSE, alone or in combination with 0.1 mg/kg of AmB. Data from two different experiments were used. (d) Kidney fungal burdens (CFU/g) each point represents mean and SD of three mice. In (d), the dotted line indicates the assay detection limit (<50 CFU) ($^{**}P < 0.01$; $^{***}P < 0.001$, AmB versus AmB + mDSE), ($\alpha = P < 0.001$, mDSE versus Infected no Tx), ($\beta = P < 0.001$, mDSE versus AmB), ($\gamma = P < 0.001$, mDSE versus AmB + mDSE), ($\delta = P < 0.05$, Infected no Tx versus AmB), ($\varepsilon = P < 0.05$, Infected no Tx versus AmB + mDSE).

with kidney fungal burden [5]. The experimental model of systemic candidiasis that we established was in line with the previously reported models.

We used this model to evaluate the efficacy of low-dose AmB supplemented with mDSE for the treatment of systemic candidiasis. Although several drugs, including echinocandins, are effective for the treatment of *C. albicans* infections, AmB is still used in many clinical settings, and the use of low-dose AmB is desirable because of the drug's toxicity. In previous studies, DLE were used in combination with antifungal drugs to treat human candidiasis [14–17], and the combination was more effective in controlling the infection than the drug alone. Our results indicate that the combined treatment of low-dose AmB plus mDSE significantly

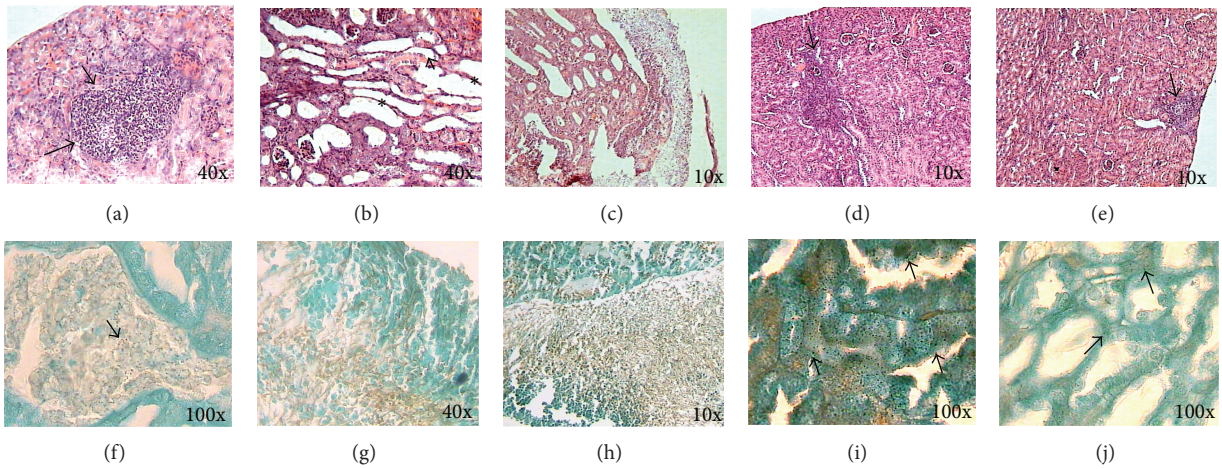

FIGURE 2: Representative histological features of kidneys from mice with systemic candidiasis. Mice were infected with 5×10^5 CFU of *Ca*07-387 and were left untreated (a), (b), (f), and (g) or were treated with 10 pg mDSE (c) and (h), 0.1 mg/kg AmB (d) and (i), or mDSE and AmB (e) and (j). Kidney sections were taken on days 2 (a) and (f) and 10 (b)–(e), (g)–(j) after infection and stained with HE (a)–(e) or with GG (f)–(j).

improved the effect of the drug, promoting an efficient control of the *Ca*07-387 strain and reducing tissue damage. Since AmB has nephrotoxic effects in humans [19], this combination would reduce the risk of toxicity associated with the administration of high doses of this drug (up to 5 mg/kg/day for 7 days in patients).

We found increased levels of IFN-γ and TGF-β1 in the kidneys of mice that received the combined treatment, compared to mice that received AmB alone. Previous studies demonstrated that Th1 responses mediated by IFN-γ resolved *C. albicans* infection [20, 21] by inducing nitric oxide and ROS production [22]. TGF-β1 limits the damage caused by excessive inflammation and promotes tissue regeneration [23]. Mice that were treated with AmB alone had higher concentrations of the pro-inflammatory cytokines TNF-α and IL-6 in their kidneys, but lower concentrations of IFN-γ, compared to mice that were treated with AmB plus mDSE. These results suggest that mice treated only with AmB have increased inflammation but lower protection from *C. albicans*.

The elimination of fungi in our experimental model correlated with high levels of serum hepcidin, which is a peptide hormone and a type II acute phase protein produced by the liver in response to iron overload and inflammatory stimuli, particularly IL-6 [24]. Hepcidin regulates the transcription of several inflammatory mediators: it binds to ferroportin, induces the activation of Jak2 and Stat3, increases the levels of SOCS3, and thus decreases the signal transduction of TLRs and cytokine receptors [25]. Hepcidin is also an antimicrobial peptide that can be detected in blood and urine, and it has direct fungicidal activity against *C. glabrata* isolates *in vitro* [26].

Serum hepcidin was significantly higher on day 8 in mice that received AmB plus mDSE, compared with mice that only received AmB; in the latter group, serum hepcidin increased its concentration only after day 15. The increased levels of serum IL-6 in mice that received the combined treatment could account for hepcidin production [24]. Mice that were treated with mDSE alone also had increased levels of serum IL-6; however, these mice did not produce hepcidin, possibly because of their high concentrations of kidney TNF-α, a negative regulator of hepcidin expression [24]. These high concentrations of kidney TNF-α might also explain why infected mice treated with mDSE alone had a higher kidney fungal burden than infected but untreated mice: the inflammation induced by this cytokine could cause tissue damage (necrosis), which would promote hyphae growth. IFN-γ upregulates hepcidin expression [27], so the increased amounts of IFN-γ in the kidneys of mice with the combined treatment could also contribute to the early production of this peptide.

Our results showed that the combination of low-dose AmB and mDSE cleared the infectious agent and at the same time reduced inflammation-associated tissue damage. During systemic candidiasis, kidney infection is associated with neutrophil infiltration [5]. The kidney damage could be caused directly by the infection, because there is a strong correlation between kidney fungal burden and serum creatinine levels [28]. However, a decreased recruitment of neutrophils to the kidney was associated with improved renal function, decreased inflammatory kidney damage, and increased survival, but it had no effect on kidney fungal burden [29]. This suggests that neutrophils (and their inflammatory mediators) are in part responsible of the tissue damage. In line with these observations, it was reported that, in patients who develop chronic disseminated candidiasis during neutrophil recovery after intensive chemotherapy, treatment with corticosteroids in addition to antifungals caused an improvement of the clinical symptoms and the resolution of the inflammatory response [30].

An inherent limitation of our study is that the intravenous infection model of systemic candidiasis represents the late

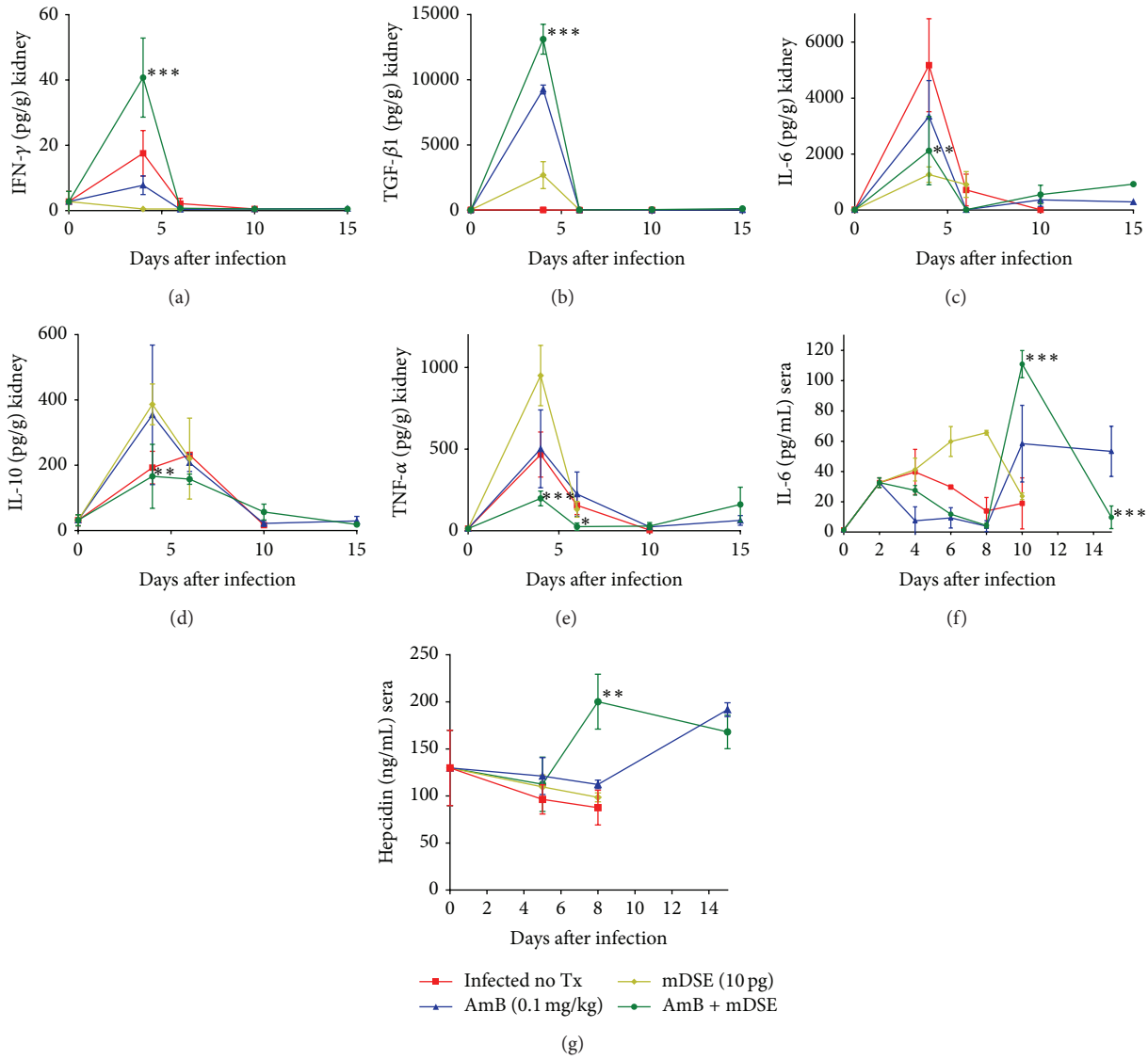

Figure 3: Effects of low-dose AmB and mDSE on kidney and serum cytokines in systemic candidiasis. Mice were infected with 5×10^5 CFU of *Ca*07-387 and treated with 10 pg of mDSE in combination with 0.1 mg/kg of AmB. (a) IFN-γ, (b) TGF-β1, (c) IL-6, (d) IL-10, and (e) TNF-α were measured in macerated kidneys. Each point represents mean and SD of three mice. (f) Serum IL-6 and (g) serum hepcidin in each experimental group: each point represents mean and SD of three mice. $^{**}P < 0.01$, $^{***}P < 0.001$, AmB versus AmB + mDSE.

stages of the disease, when the fungal cells are already in the bloodstream. It does not address the early stages of the disease (i.e., translocation of *C. albicans* from the gut), which would explain how *C. albicans* reaches the blood. However, we provide evidence that indicates that the combination of low-dose AmB with human DLE could have appropriate efficacy and safety as a treatment for systemic candidiasis.

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Research Article

Allergen-Specific IgG Antibodies Purified from Mite-Allergic Patients Sera Block the IgE Recognition of *Dermatophagoides pteronyssinus* Antigens: An *In Vitro* Study

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One of the purposes of specific immunotherapy (SIT) is to modulate humoral immune response against allergens with significant increases in allergen-specific IgG levels, commonly associated with blocking activity. The present study investigated *in vitro* blocking activity of allergen-specific IgG antibodies on IgE reactivity to *Dermatophagoides pteronyssinus* (Dpt) in sera from atopic patients. Dpt-specific IgG antibodies were purified by ammonium sulfate precipitation followed by protein-G affinity chromatography. Purity was checked by SDS-PAGE and immunoreactivity by slot-blot and immunoblot assays. The blocking activity was evaluated by inhibition ELISA. The electrophoretic profile of the ammonium sulfate precipitated fraction showed strongly stained bands in ligand fraction after chromatography, compatible with molecular weight of human whole IgG molecule. The purity degree was confirmed by detecting strong immunoreactivity to IgG, negligible to IgA, and no reactivity to IgE and IgM. Dpt-specific IgG fraction was capable of significantly reducing levels of IgE anti-Dpt, resulting in 35%–51% inhibition of IgE reactivity to Dpt in atopic patients sera. This study showed that allergen-specific IgG antibodies purified from mite-allergic patients sera block the IgE recognition of *Dermatophagoides pteronyssinus* antigens. This approach reinforces that intermittent measurement of serum allergen-specific IgG antibodies will be an important objective laboratorial parameter that will help specialists to follow their patients under SIT.

1. Introduction

Allergic diseases are considered one of the major health problems worldwide and constitute a breakdown in the immune tolerance against natural exposure to environmental antigens [1]. Among them, the house dust mites (HDMs) from the family Pyroglyphidae, mostly *Dermatophagoides pteronyssinus*, play an important role to induce respiratory allergic diseases, particularly asthma and allergic rhinitis, in genetically predisposed individuals [2, 3]. Recent data from World Allergy Organization (WAO) show that the prevalence of allergic diseases has occurred in 30%–40% of the world population, of which 400 million suffer from allergic rhinitis [4]. The immediate symptoms of allergic rhinitis are caused by allergen-induced crosslinking of mast cell-bound IgE antibodies and release of inflammatory mediators as histamine and leukotrienes [5].

Several studies have been performed focusing on the development of new treatments beyond the pharmacotherapy already established, aiming to relieve the symptoms caused by exacerbated responses of the organism against allergens [6]. Allergen-specific immunotherapy (SIT) is the main treatment used for allergy and involves mechanisms that include the production of blocking antibodies, the shifts toward

Th1 response, and tolerance induction [7, 8]. IgG antibodies induced by SIT may act as blocking agents by competing with IgE for allergen binding, inhibiting the activation of IgE-dependent mast cells and basophils and reducing IgE-mediated allergic inflammation [5, 9]. Previous studies have attributed to IgG subclasses, particularly IgG4, a protective activity, by acting like an inhibition factor of IgE-mediated hypersensitivity reaction after long-time antigen exposure. Consequently, IgG4 antibodies might neutralize allergens or block IgE binding to allergens, attenuating thereby the allergic reaction [10, 11]. Even though SIT induces high levels of specific IgG1 and IgG4 [12], the blocking capacity of these antibody classes remains to be clarified. Thus, the knowledge about the role and mechanisms of blocking activity of these antibodies can facilitate the progress and development of new techniques for SIT [13].

Considering that a successful SIT correlates with decreasing of clinical symptoms and increasing of allergen-specific IgG antibody levels, the aim of this study was to investigate *in vitro* the blocking capability of specific IgG antibodies purified from mite-allergic patients sera on the IgE reactivity to *D. pteronyssinus*.

2. Materials and Methods

2.1. Subjects and Skin Prick Test. Thirty-six patients, male and female, aged 18 to 60, with clinical history of allergic rhinitis (atopic group) were recruited from the Laboratory of Allergy and Clinical Immunology, Federal University of Uberlandia, Uberlandia, MG, Brazil. As inclusion criteria, patients should have positive skin prick test (SPT) to at least *Dermatophagoides pteronyssinus* (Dpt) allergen extract from a panel of standardized aeroallergens (FDA Allergenic Ltda, Rio de Janeiro, RJ, Brazil) as follows: house dust mites (*D. pteronyssinus*, *D. farinae, and Blomia tropicalis*); cockroaches (*Blattella germanica* and *Periplaneta americana*); mold (*Alternaria alternata*); and pet danders (*Felis domesticus* and *Canis familiaris*). The exclusion criteria were positive results in rheumatoid factor assay (Bio Látex FR, Bioclin, Belo Horizonte, MG, Brazil), the use of antihistamines in the previous week to the skin test, and previous or current immunotherapy.

Fifteen volunteers, healthy subjects, male and female, aged 18 to 60, were selected based on the absence of clinical history or symptoms of allergic rhinitis and negative SPT to a panel of standardized aeroallergens (nonatopic group). In parallel, blood samples (10 mL) were collected from all individuals, and the serum was stored at −20°C until serological assays.

The study was approved by the Ethics Committee in Human Research of the Federal University of Uberlandia, and written informed consent was obtained from all participants.

2.2. Measurement of D. pteronyssinus-Specific IgE, IgG1, and IgG4. All serum samples were assessed by enzyme linked immunosorbent assay (ELISA) for measuring levels of IgE, IgG1, and IgG4 to Dpt as previously described [14, 15], with some modifications. Briefly, microtiter plates were coated with Dpt extract (2 μg/well; Hollister-Stier Laboratories, Spokane, WA, USA), blocked with phosphate-buffered saline (PBS, pH 7.2) containing 0.05% Tween 20 and 1% bovine serum albumin (PBS-T-BSA) for IgE and PBS-T-BSA 0.1% for IgG1 and IgG4, and then incubated with serum samples diluted 1 : 2 (IgE), 1 : 10 (IgG1), or 1 : 5 (IgG4) for 2 hours at 37°C. After washing, plates were incubated with biotinylated secondary antibodies as anti-human IgE (1 : 1,000; Kirkegaard and Perry Laboratories Inc. (KPL), Gaithersburg, MD, USA), anti-human IgG1 (1 : 3,000; Sigma Chemical Co., St Louis, MO, USA), or anti-human IgG4 (1 : 1,000; Sigma) for 1 hour at 37°C and subsequently with streptavidin peroxidase (1 : 1,000; Sigma). The assay was developed with ABTS-peroxidase substrate system (KPL), and optical density (OD) values were determined at 405 nm. Antibody levels were expressed as ELISA index (EI) according to the following formula: EI = OD test sample/cutoff, where the cutoff was established as the mean OD value of negative control sera plus 3 standard deviations. EI values >1.2 were considered to be positive in order to exclude borderline reactivity values close to EI = 1.0.

2.3. Serum Pools and Salting-Out Precipitation. Five serum samples of each patient group were selected to constitute the Dpt-specific (atopic) and nonspecific (nonatopic) serum pools. The selection criteria were based on the highest (EI > 2.0) and lowest (EI < 1.0) values of reactivity to both IgG1 and IgG4 to Dpt allergen in atopic and nonatopic groups, respectively. Thus, these criteria would allow obtaining a maximal diversity in antigen/epitope recognition, according to different seroreactivity profiles and intensity of reaction observed in immunoenzymatic assays, favouring the identification of biological phenomena rather than individual immune responses. Initially, the serum albumin from specific and nonspecific pools was partially depleted by salting-out precipitation using 40% ammonium sulfate [16]. The supernatant (S 40%) and precipitated (P 40%) fractions obtained were dialyzed and concentrated by using Amicon system (Millipore, Billerica, MA, USA) and further analyzed in polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) on 8% gels under nonreducing conditions. Samples were solubilized in sample buffer, boiled at 96°C for 5 min, and applied to the gel in parallel with molecular weight markers (BenchMark Protein Ladder, Invitrogen, Carlsbad, CA, USA). Protein profile was visualized with blue silver staining [17].

2.4. Slot-Blot Assays. Nitrocellulose membranes (0.45 μm; Bio-Rad Laboratories Inc., Hercules, CA, USA) were coated with serum pools, S 40% and P 40% fractions, and bovine serum albumin (BSA) as negative control, using the Mini Protean II Multiscreen Apparatus (Bio-Rad). After blocking with PBS-T plus 5% skim milk, membranes were incubated with detection antibodies labeled with peroxidase (for IgG diluted 1 : 5,000 and IgM diluted 1 : 5,000; Calbiochem Merck, Darmstadt, Germany), biotin (for IgA diluted 1 : 10,000; Sigma), and monoclonal antibody to IgE (1 : 1,000; Sigma). After incubation with streptavidin peroxidase (1 : 1,000; Sigma) or anti-mouse IgG/peroxidase (1 : 1,000; Oncogene Science, Cambridge, MA, USA), when appropriate, the membranes were revealed with DAB tablets (Sigma). Bands were analyzed

using the ImageJ 1.46 software (National Institute of Mental Health, Bethesda, MA, USA).

2.5. Purification of Total IgG by Affinity Chromatography and Immunoblots. The P 40% fractions of each specific and nonspecific serum pool were loaded into affinity chromatography columns (Pierce Protein G Agarose, Thermo Fisher Scientific Inc., Rockford, IL, USA), previously equilibrated with binding buffer (0.02 M phosphate buffer, pH 8.0). Samples were diluted 1 : 1 in binding buffer, applied to the column, and washed with at least 10 volumes of binding buffer. Total IgG was eluted in elution buffer (0.1 M glycine, pH 2.6), and 1 mL fractions were collected. After pH neutralization with 1 M Tris-HCl, pH 9.0, absorbance was read at 280 nm. Values of absorbance and pH of each fraction prior to neutralization were used to build the chromatographic profile.

The purity of total IgG obtained from the affinity chromatography was checked using SDS-PAGE on 8% gels as described above. To confirm the immunoreactivity of the eluted fractions, immunoblots were performed for detection of IgG, IgA, IgE, and IgM antibodies. Briefly, the fractions previously separated on 8% SDS-PAGE were electrotransferred onto nitrocellulose membranes, and blotting efficiency was validated by reversible Ponceau S staining. Membranes were blocked with 5% skim milk in PBS-T. After blocking, blots were incubated with the respective detection antibodies to IgG, IgA, IgE, and IgM as described in slot-blot assays and revealed with DAB.

After monitoring the eluted fractions by 8% SDS-PAGE, the samples containing IgG were pooled, dialyzed, and concentrated against PBS by using Amicon system. Protein concentration was determined using the Lowry method [18].

2.6. Determination of Optimal Concentrations of Specific and Nonspecific IgG Fractions. An indirect ELISA was carried out to determine the optimal concentrations of specific and nonspecific IgG fractions through detection of IgG1 and IgG4 anti-Dpt as well as residual specific IgE as previously described [14, 15], with modifications. Briefly, plates were coated with Dpt (2 μg/well), blocked with PBS-T-BSA 0.1%, and subsequently incubated with specific and nonspecific IgG fractions diluted from 40 to 2.5 μg/well. Subsequent steps were similar to the ELISA for detection of Dpt-specific IgE, IgG1, and IgG4 as described above.

2.7. Inhibition ELISA. To evaluate the blocking activity of the Dpt-specific IgG fractions on IgE reactivity to Dpt allergen, an inhibition ELISA was developed by using three serum pools of atopic patients with different positivity for Dpt-specific antibody classes as follows: pool I (IgE+, IgG1+, and IgG4–); pool II (IgE+, IgG1–, and IgG4+); and pool III (IgE+, IgG1+, and IgG4+). Briefly, plates were coated with Dpt (2 μg/well), blocked with PBS-T-BSA, and incubated with the optimal concentration of specific or nonspecific IgG fractions for 1 h at 37°C. Then, the serum pools from atopic patients were diluted 1 : 2, incubated for 1 h at 37°C, and followed by incubation with biotinylated anti-human IgE (1 : 1,000; KPL). Subsequent steps were similar to the ELISA for detection of IgE anti-Dpt as described above. Results were reported as absorbance values at 405 nm and inhibition percentage as follows: % inhibition = 1 − (DO inhibited/DO uninhibited) × 100 [19].

2.8. Statistical Analysis. Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software Inc.). Comparison between levels of IgE, IgG1, and IgG4 antibodies to Dpt within the groups was analyzed by the Mann-Whitney test. Differences in slot-blot data were determined by Student's t-test. Differences between the groups were analyzed by one-way ANOVA using the Bonferroni posttest (IgE reactivity and inhibition ELISA). Correlation between the levels of antibody classes was analyzed by the Spearman correlation test. Values of $P < 0.05$ were considered statistically significant.

3. Results

The demographic and clinical characteristics of the study subjects are shown in Table 1. All patients from the atopic group had clinical history of allergic rhinitis related to HDMs exposure and positive SPT to aeroallergen extracts, with higher concomitant sensitization to HDMs, *D. pteronyssinus,* and *D. farinae* (97%) than to *Blomia tropicalis* (64%) and to other aeroallergens (<54%) ($P < 0.0001$). The atopic and nonatopic groups were comparable regarding the sex and age.

Levels of IgE to *D. pteronyssinus* were higher in atopic patients than in nonatopic subjects ($P < 0.0001$; Figure 1(a)), with 87% of positivity in atopics and no positivity in nonatopics. Likewise, levels of IgG1 anti-Dpt were higher in atopics than nonatopics ($P < 0.05$), although the positivity was similar between groups. In contrast, levels and positivity of IgG4 anti-Dpt were similar between the groups. Significant positive correlations were found between Dpt-specific IgE and IgG1 ($r_S = 0.5815$; $P = 0.0002$) or IgG4 ($r_S = 0.3926$; $P = 0.0179$), with slightly higher number of double-positive patients for IgE and IgG4 (65%) than for IgE and IgG1 anti-Dpt (56%) (Figure 1(b)).

To select the serum samples with the highest and lowest concomitant IgG1 and IgG4 reactivity within the atopic and nonatopic groups, respectively, levels of IgG1 and IgG4 were compared as shown in Figure 1(c). Five serum samples were selected within each group and pooled to constitute the Dpt-specific and nonspecific serum pools, respectively. The Dpt-specific IgE, IgG1, and IgG4 reactivity profiles in each serum pool revealed mean EI values above 4.2 for the three antibody classes in the atopic group and below 1.0 in the nonatopic group (Table 2).

Total human IgG purification was performed in two steps. Firstly, Dpt-specific and nonspecific serum pools were partly purified by 40% ammonium sulfate precipitation, obtaining the S 40% and P 40% fractions. The immunoglobulin profile in these fractions was verified by slot-blot, showing that all analyzed classes (IgG, IgA, IgE, and IgM) were more concentrated in P 40% than S 40% fractions ($P < 0.01$) as shown in Figures 2(a)–2(b).

Secondly, the P 40% fractions of each serum pool were loaded into protein G-agarose column, and a representative chromatogram is illustrated in Figure 3(a). The peak I (tubes

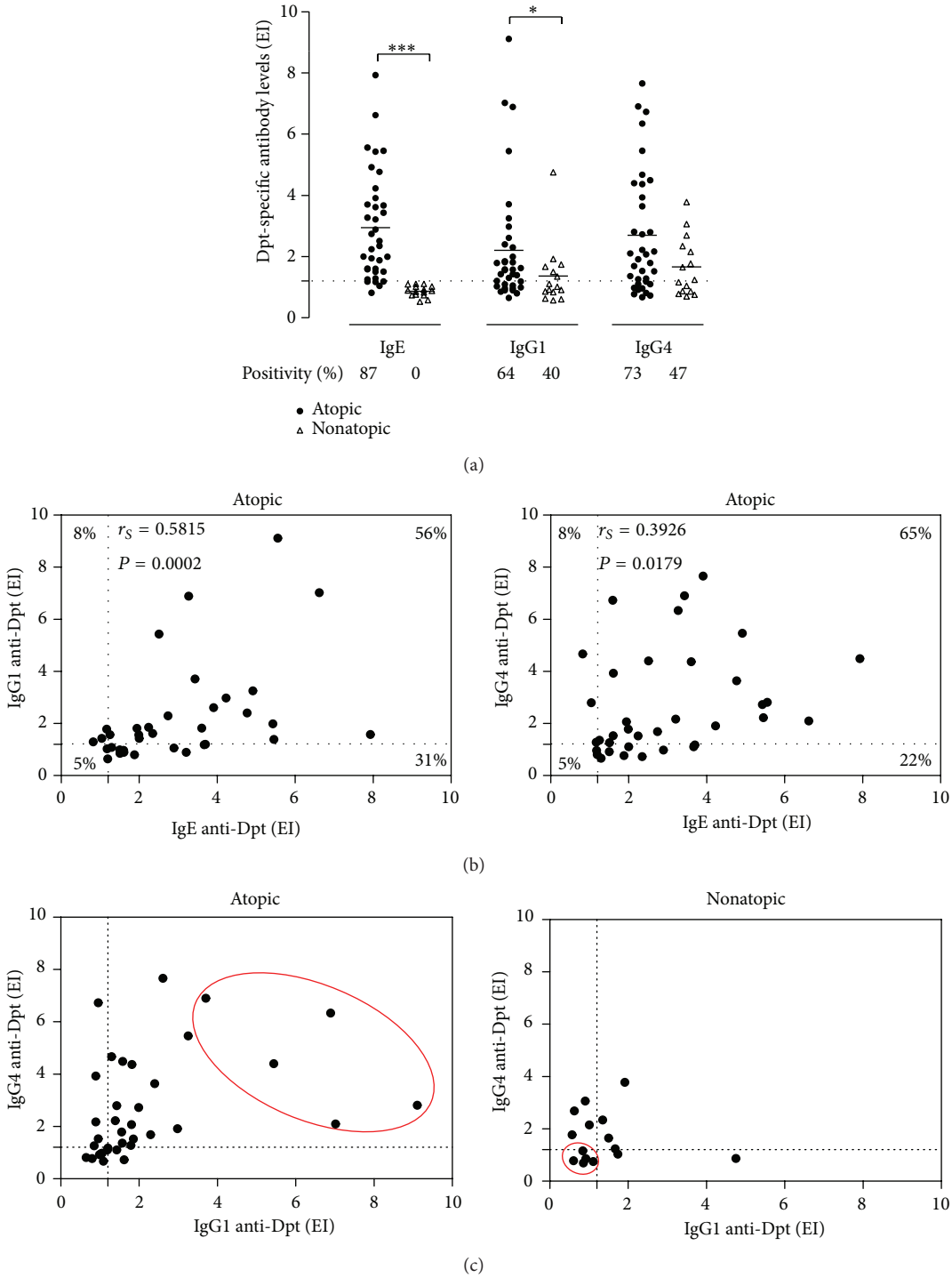

FIGURE 1: (a) Levels of IgE, IgG1, and IgG4 antibodies to *Dermatophagoides pteronyssinus* (Dpt) allergen extract in serum samples from atopic and nonatopic patients. Data are expressed in ELISA index (EI), and mean is indicated by horizontal bars. The dashed line indicates the cutoff of the reaction (EI > 1.2). Percentages of positive samples are also indicated. Statistically significant differences were determined by the Mann-Whitney test ($^{*}P < 0.05$; $^{***}P < 0.0001$). (b) Correlation between levels of Dpt-specific IgE versus IgG1 and IgE versus IgG4 anti-Dpt in serum samples from atopic patients. Percentages of double positive, double negative, or single positive for each antibody class are indicated in the correspondent corners. Spearman's correlation coefficient and statistical significance are also indicated. (c) Comparison between levels of IgG1 and IgG4 anti-Dpt in serum samples from atopic and non-atopic patients. Five serum samples (red ellipses) of each patient group were selected to constitute the specific (atopic) and nonspecific (nonatopic) total IgG pools. The dashed lines indicate the cutoff of the reaction (EI > 1.2).

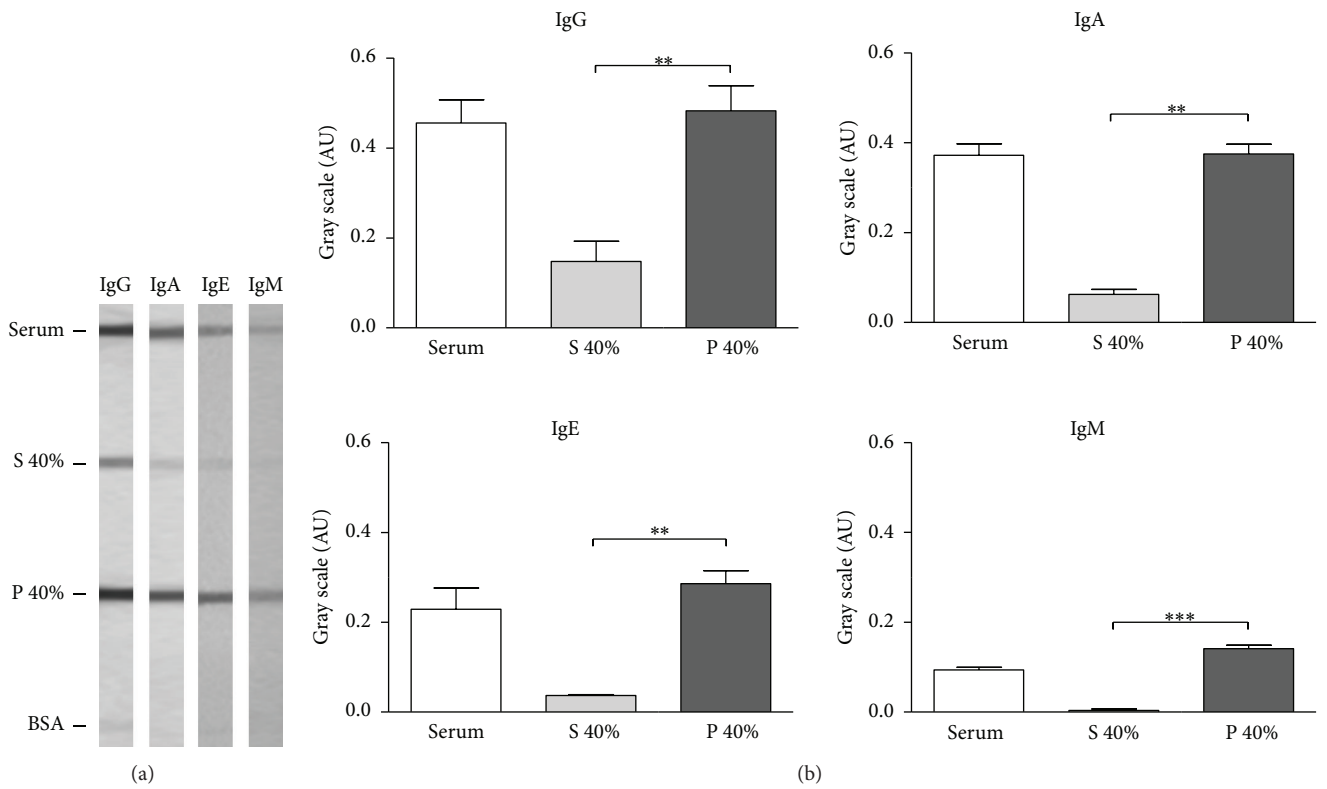

Figure 2: (a) Slot-blots showing reactivity for IgE, IgA, IgG, and IgM in the serum, supernatant (S 40%), and precipitated (P 40%) fractions obtained from precipitation of the serum with 40% ammonium sulfate solution, and bovine serum albumin (BSA) as irrelevant protein. (b) Slot-blot data analysis by measuring the intensity of the bands in gray scale and expressed in arbitrary units (AU). Statistically significant differences were determined by Student's t test ($^{**}P < 0.01$; $^{**}P < 0.001$).

Table 1: Demographic and clinical characteristics of the study subjects.

Characteristics	Groups		P value
	Atopic	Nonatopic	
Number of subjects	36	15	—
Age (year)			
Mean ± SD	24.6 ± 5.8	28 ± 11.64	0.1725[a]
Sex (M : F)	13 : 23	2 : 13	0.1770[b]
Positive skin prick test (n, %)			
Dermatophagoides pteronyssinus	36 (100%)	0	<0.0001[b]
Dermatophagoides farinae	35 (97%)	0	<0.0001[b]
Blomia tropicalis	23 (64%)	0	<0.0001[b]
Blatella germanica	10 (28%)	0	0.0009[b]
Periplaneta americana	9 (25%)	0	0.0022[b]
Alternaria alternata	2 (6%)	0	0.4930[b]
Felis domesticus	19 (53%)	0	<0.0001[b]
Canis familiaris	14 (39%)	0	<0.0001[b]

[a]Student's t-test; [b]Fisher's exact test ($P < 0.05$); SD: standard deviation.

3 to 6) was obtained during washing with binding buffer, representing the nonligand fraction (NLF). The peak II (tubes 29 to 34) was obtained after elution buffer, corresponding to the ligand fraction (LF). To check the purification of these fractions, SDS-PAGE 8% was performed. A representative electrophoretic profile shows strongly stained bands around 160 kDa in the LF fractions, compatible with the molecular weight of whole IgG molecules, including the high and light chains (Figure 3(b)). The immunoreactivity of these LF fractions was verified by immunoblots, showing a

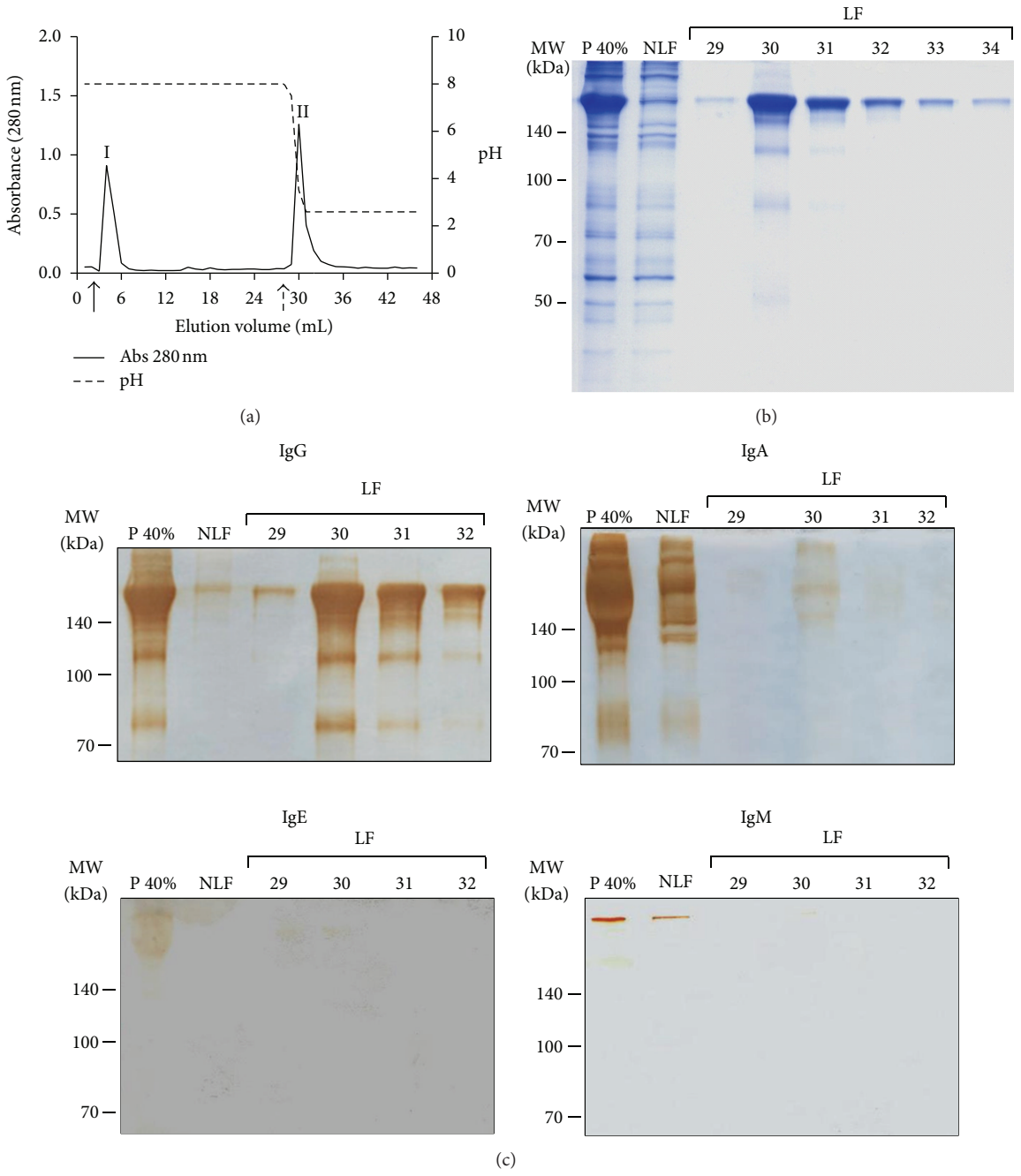

Figure 3: (a) Representative chromatogram of the total human IgG purification by affinity chromatography in protein G-agarose obtained from the 40% ammonium sulfate precipitated fraction (P 40%) of a serum pool. I—elution peak of P 40% nonligand fraction after washing with 0.02 M phosphate buffer pH 8.0 (black arrow); II—elution peak of P 40% ligand fraction after washing with 0.1 M glycine buffer pH 2.6 (dashed arrow). Data are expressed in absorbance (280 nm). Elution volume consisted of 1 mL in each tube. Values of pH were also measured in each elution tube. (b) Electrophoretic profile in SDS-PAGE 8% stained with blue silver. NLF—nonligand fraction, LF—ligand fraction, corresponding to the tubes 29–34. Markers of molecular weight (MW) are indicated on the left in kilodaltons (kDa). (c) Immunoblots for detection of IgG, IgA, IgE, and IgM in the serum fractions after purification in protein G-Agarose as shown in (b). Bands were revealed with DAB as described in Methods.

strong reactivity to IgG, whereas IgA reactivity was negligible, and no reactivity was detected to IgE and IgM antibodies (Figure 3(c)).

To determine the optimal concentration of Dpt-specific and nonspecific IgG fractions to be used in inhibition ELISA, an indirect ELISA was performed to detect levels of IgG1 and IgG4 anti-Dpt in these fractions. As shown in Figures 4(a)–4(c), the best distinction between the two fractions was found when 40 μg/well was used, considering the ratio of reactivity between specific and nonspecific fractions as well

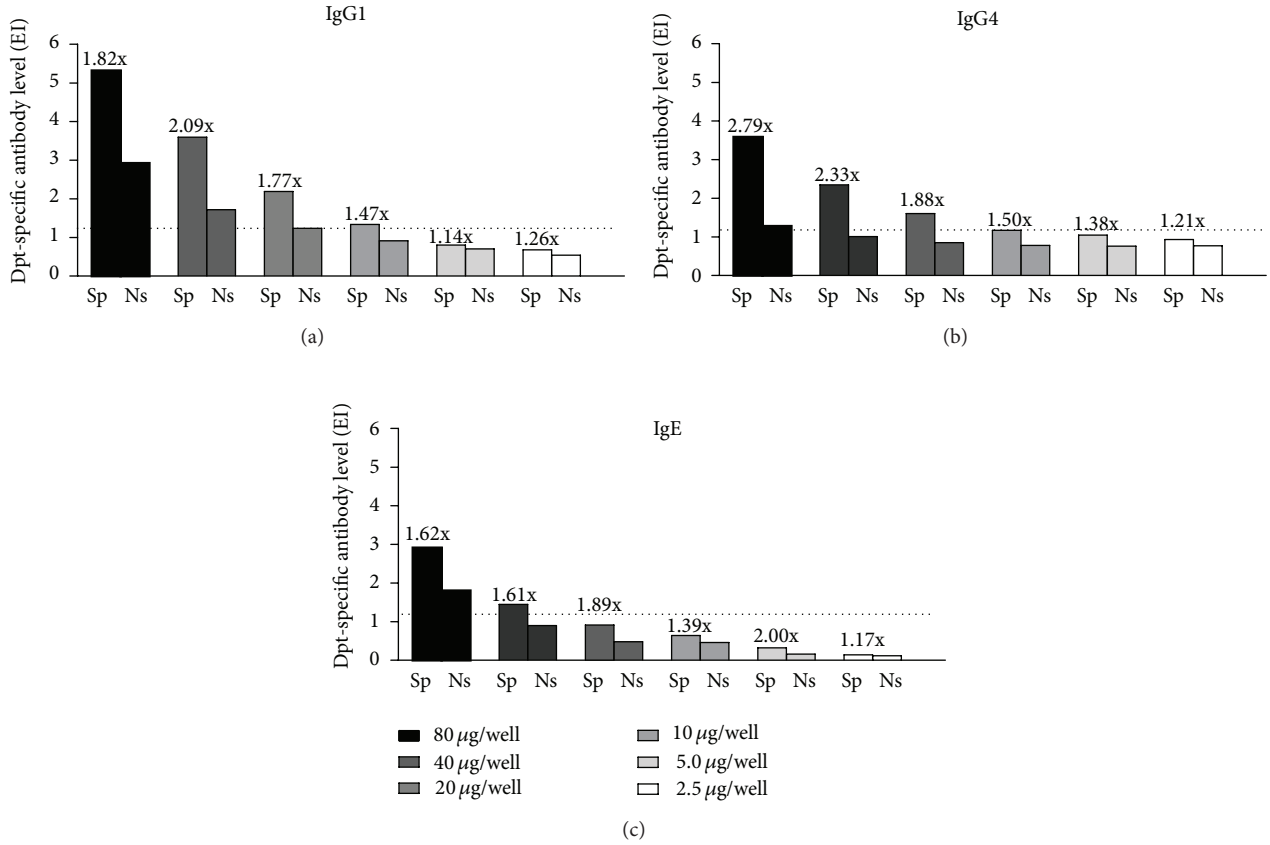

Figure 4: Levels of IgG1 (a), IgG4 (b), and IgE (c) antibodies to *Dermatophagoides pteronyssinus* (Dpt) allergen extract in the specific (Sp) and nonspecific (Ns) purified IgG fractions obtained from atopic and nonatopic patients, respectively, determined by ELISA. Purified IgG fractions were titrated at two-fold dilutions from 80 to 2.5 μg/well, and data are expressed in ELISA index (EI). The dashed lines indicate the cutoff of the reaction (EI > 1.2). The values indicating the Sp/Ns ratio for each antibody class and analyzed concentration are also indicated.

Table 2: *Dermatophagoides pteronyssinus*-specific IgE, IgG1, and IgG4 reactivity profile in serum pools of atopic ($n = 5$) and nonatopic ($n = 5$) subjects.

Dpt-specific antibody levels	Groups	
	Atopic	Nonatopic
	Dpt-specific serum pool	Nonspecific serum pool
IgE (mean EI ± SD)	4.28 ± 1.73	1.06 ± 0.54
IgG1 (mean EI ± SD)	6.43 ± 2.00	0.85 ± 0.17
IgG4 (mean EI ± SD)	4.51 ± 2.11	0.85 ± 0.19

Dpt: *Dermatophagoides pteronyssinus* allergen extract; EI: ELISA index; SD: standard deviation.

as the cutoff value for each reaction. However, we could not determine the saturating concentration to occupy all the sites because the reaction background generated by nonspecific IgG fractions also increased considerably at 80 μg/well for the three antibody classes, particularly for IgG1 and IgE. It is noteworthy that IgE reactivity detected in both specific and nonspecific IgG fractions at 40 μg/well was borderline or below the cutoff (Figure 4(c)). For these reasons, the concentration of 40 μg/well was chosen for further experiments.

To verify which is the predominant antibody subclass in the purified specific IgG fraction at 40 μg/well, the IgG1/IgG4 ratio was calculated (IgG1/IgG4 = 1.53), showing that the predominant antibody subclass in purified specific IgG fractions was IgG1, as expected.

Next, IgE reactivity to Dpt in the presence or absence of specific or nonspecific IgG fractions was determined by inhibition ELISA using three serum pools (I, II, and III) of atopic patients selected with basis on the positivity for the three antibody classes: pool I (IgE+, IgG1+, and IgG4−); pool II (IgE+, IgG1−, and IgG4+); and pool III (IgE+, IgG1+, and IgG4+) (Table 3). For the three serum pools tested, the presence of specific IgG fraction significantly reduced IgE reactivity to Dpt when compared to PBS ($P < 0.0001$) (Figure 5(a)). As shown in Table 3, the levels of Dpt-specific IgE detected in the pool II were lower than those found in the pools I and III. Thus, the reactivity of PBS control in the pool II was lower than in pools I and III, but with no significant differences between them (Figure 5(a)). A similar effect of IgE reduction was observed in the presence of nonspecific IgG fractions ($P < 0.0001$). However, the IgE reactivity was significantly lower in the presence of specific fractions than in nonspecific IgG fractions ($P < 0.0001$). When the percentage of inhibition for IgE reactivity was evaluated, the specific IgG fractions

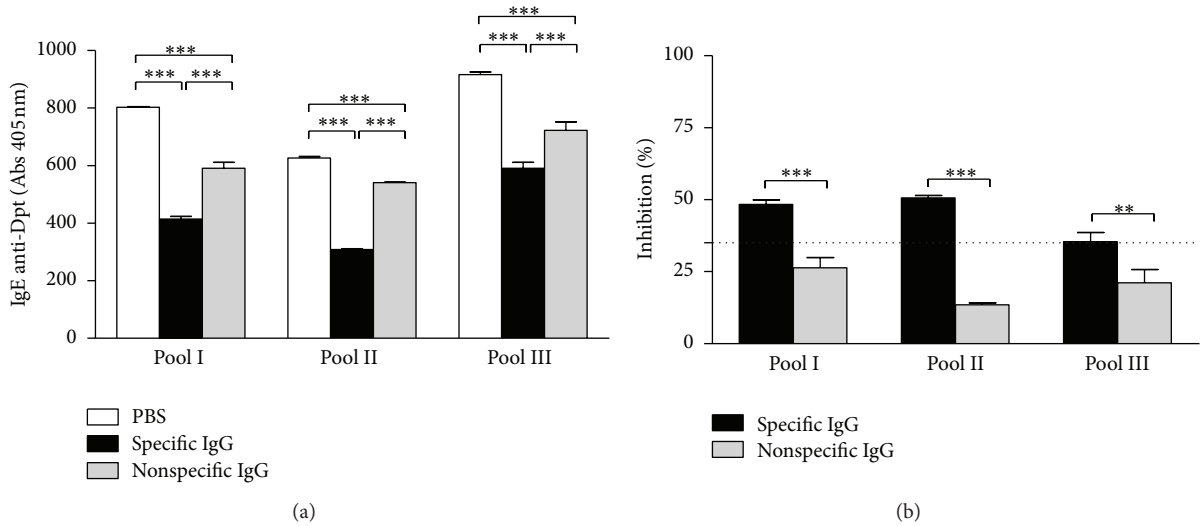

Figure 5: Inhibition ELISA results showing the blocking capacity of specific and nonspecific purified IgG antibodies for IgE reactivity to *Dermatophagoides pteronyssinus* (Dpt) allergen extract in serum pools of atopic patients. Three serum pools (I, II, and III) with different positivity for antibody classes were used as follows: pool I (IgE+, IgG1+, and IgG4−); pool II (IgE+, IgG1−, and IgG4+); and pool III (IgE+, IgG1+, and IgG4+). (a) Levels of IgE anti-Dpt expressed in absorbance (405 nm). Statistically significant differences were determined by one-way ANOVA and the Bonferroni posttest ($^{***}P < 0.0001$). (b) Percentage of inhibition of IgE binding by blocking specific and nonspecific IgG antibodies in three serum pools of atopic patients. The dashed line indicates a threshold inhibition value of 35%. Statistically significant differences were determined by Student's *t*-test ($^{**}P < 0.01$; $^{***}P < 0.0001$).

Table 3: *Dermatophagoides pteronyssinus*-specific IgE, IgG1, and IgG4 reactivity profile in three serum pools (I, II, and III) of atopic patients.

Dpt-specific antibody levels	Atopic serum pools		
	I	II	III
IgE (mean EI ± SD)	**4.46 ± 3.63**	**1.80 ± 0.70**	**4.20 ± 2.55**
IgG1 (mean EI ± SD)	**1.87 ± 0.60**	0.90 ± 0.06	**3.23 ± 2.06**
IgG4 (mean EI ± SD)	0.93 ± 0.22	**2.81 ± 2.17**	**4.35 ± 2.24**

Dpt: *Dermatophagoides pteronyssinus* allergen extract; EI: ELISA index; SD: standard deviation; positive values are represented in bold.

were able to inhibit ≥50% IgE reactivity for the pools I and II, and above 35% inhibition for the pool III (Figure 5(b)).

4. Discussion

It is known that the final purpose of allergen-specific immunotherapy is to modulate the immunological profile against allergens and that the benefits achieved are long-lasting even though the therapy is discontinued [20]. Increases in allergen-specific IgG1 and IgG4 levels are associated with blocking activity by preventing IgE binding to the allergen and consequently leading to a reduction of the allergic inflammatory response [10–12].

The characterization, quantification, and evaluation of the blocking capability of IgG antibodies on the allergen-IgE interaction using *in vitro* tests represent a main tool to elucidate the role of different types of immunoglobulins in the allergic response. In the present study, this approach was performed initially through purification of Dpt-specific IgG fractions from serum pools of mite-allergic patients and subsequently, evaluation of its blocking capability on the IgE reactivity by inhibition ELISA assays.

The recruitment of participants for the atopic group of this study was based on clinical history of allergic rhinitis and positive SPT to at least Dpt allergen extract. A previous study in patients of the Triângulo Mineiro region, Brazil [21], showed high positivity percentages to HDM extracts, underlining *D. pteronyssinus* and *D. farinae* as relevant sensitizing agents in this region. IgE levels and seropositivity in atopic patients found in this study resembled those found in our previous work evaluating the levels of IgE, IgA, and IgG4 antibodies to *D. pteronyssinus* and to its major allergens, Der p1 and Der p2, in samples of serum and saliva from allergic and nonallergic children [14]. Although the mean levels of IgG4 to Dpt were higher in atopic than in nonatopic subjects of the present study, this difference was not statistically significant as that found in our previous studies with *D. pteronyssinus* [14] and *Blomia tropicalis* [22], reinforcing that antigens that induce IgE antibodies are also good inducers of IgG4 antibodies. These findings were supported by significant positive correlation found between Dpt-specific IgE and IgG4 antibodies in atopic patients. Likewise, levels of Dpt-specific IgG1 antibodies were higher in atopic than in nonatopic subjects similarly to our findings of IgG1 anti-*B. tropicalis* [22], supporting that IgG1 antibodies might be more closely related to allergen exposure. Also, the significant positive correlation found between Dpt-specific IgE and IgG1 antibodies reinforces these data. There are ongoing discussions on whether IgG4 is a blocking or an anaphylactic antibody and whether IgG1 is associated with exposure and protective role [23, 24].

Considering the highest and lowest concomitant IgG1 and IgG4 reactivities to Dpt in atopic and nonatopic patients, respectively, serum samples were selected to obtain the specific and nonspecific serum pools. These serum pools were partially purified by salting-out precipitation using 40% ammonium sulfate and then submitted to affinity chromatography to get the specific and nonspecific IgG fractions. These techniques of immunoglobulin purification have been widely used with high quality and integrity of recovered antibodies [25, 26]. The use of the ammonium sulfate precipitation allows obtaining high immunoglobulin concentration with considerable level of purity and no damage to its functional activity. The electrophoretic profile after 40% ammonium sulfate precipitation of the specific and nonspecific serum pools showed an enrichment of high molecular weight proteins in the precipitated fraction and a broad depletion of human serum albumin, consistently with the literature data [27]. In addition, all analyzed antibody classes were more concentrated in the precipitated fractions. Using this technique, we were able to optimize the protein G-agarose affinity chromatography, avoiding interferences from other serum proteins, especially serum albumin.

The electrophoretic profile after affinity chromatography showed strongly stained bands in the ligand fractions, compatible with the molecular weight of the human whole IgG molecule. Although other bands were visible, they can be considered degradation products since they were stained in immunoblot assays for detection of total IgG. The purification was considered successful because a strong reactivity to IgG was detected, whereas IgA reactivity was negligible, and no reactivity was found for IgE and IgM. Although recent studies have been looking for new clarifications concerning the role of IgD in regulation of immune system [28, 29], we did not perform immunoblots for IgD since its serum concentration is despicable when compared with the other immunoglobulins [30].

It is well known that SIT leads to an increase in the allergen-specific IgG production [31–33], with the ability to block the IgE-allergen interaction as well as interaction of these complexes with B cells and basophil activation [34]. Several studies have correlated high IgG serum levels, especially IgG1 and IgG4, with clinical responses of patients after SIT [11, 35, 36]. The association of IgG4 with protective activity is related to its function as a blocking antibody or a marker of tolerance induction, resulting in a decreased sensitivity of T cells and consequently in a suppression of the late-phase reactions [37]. As a marker of tolerance induction, IgG4 antibody measurements may be particularly valuable in follow-up studies, where a considerable increase in IgG4 levels can be a strong indicator of the activation of tolerance-inducing mechanisms [38].

In the present study, we obtained Dpt-specific IgG fractions purified from serum pools of atopic patients that were not under any immunotherapy. In addition, the IgG1 and IgG4 levels to Dpt were measured in these fractions to warrant the presence of high levels of these specific blocking antibodies. A low level of residual IgE antibody was detected in the chosen concentration of specific IgG fraction, suggesting a probable crossreactivity with the biotinylated anti-human IgE antibody used in ELISA, since no IgE reactivity was detected in this purified IgG fraction in immunoblot assays using another secondary antibody.

The blocking activity of the allergen-specific IgG fraction on the IgE reactivity to Dpt allergen extract was then evaluated by inhibition ELISA. Allergen-specific IgG fraction was capable of reducing levels of IgE anti-Dpt, resulting in 35%–51% inhibition of IgE reactivity to Dpt in the three serum pools tested. Also, we verified that the presence of specific IgG1 or IgG4 or both subclasses together with specific IgE in the tested serum pools did not interfere with the IgE blocking capability of these specific IgG fractions. Considering that the inhibition phenomena of IgE binding seen for the specific IgG fractions include both IgG1 and IgG4 subclasses, it was not possible to attribute a more protective role to IgG4 compared to IgG1 in the design of the present study. However, as the IgG1/IgG4 ratio in the specific IgG fractions was higher than 1.0, it may be speculated that the specific IgG1 antibody could also play a protective role. Further studies should be conducted using absorption methods or purification of specific IgG1 or IgG4 fractions from patients that are single positive for each IgG subclass to evaluate separately the role of each subclass of specific IgG fraction. This blocking role of allergen-specific IgG antibodies has been recently investigated in mouse models through passive immunization with specific IgG antibodies for prevention and treatment of allergy to major birch and grass pollen allergens [39]. The authors showed that mice treated with anti-Phl p 1 IgG after sensitization with rPhl p 1 allergen had reduced Phl p 1-specific IgE levels in all time points tested. Also, inhibition percentages of IgE binding to Bet v 1 (23.8% to 57.4%) and Phl p 1 (30.8 to 63.3%) were found in the groups treated with the respective allergen-specific IgG antibodies [39]. In our study, when nonspecific IgG fraction was used, we also observed a decrease in Dpt-specific IgE levels, although with minor inhibition, which could be associated with the heterogeneity of the allergen composition of mite extracts [40, 41]. In this context, IgG antibodies could react with both allergenic and non-allergenic components present in the crude allergen extract, but only those IgE epitope-specific IgG antibodies belong to the true blocking IgG antibodies [41].

Altogether, our results showed that allergen-specific IgG antibodies purified from mite-allergic patient sera using available and standardized methodology are able to inhibit IgE reactivity to Dpt allergen extract. This approach reinforces that the intermittent measurement of serum allergen-specific IgG antibodies will be an important objective laboratorial parameter that will help specialists to follow their patients under allergen-specific immunotherapy.

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