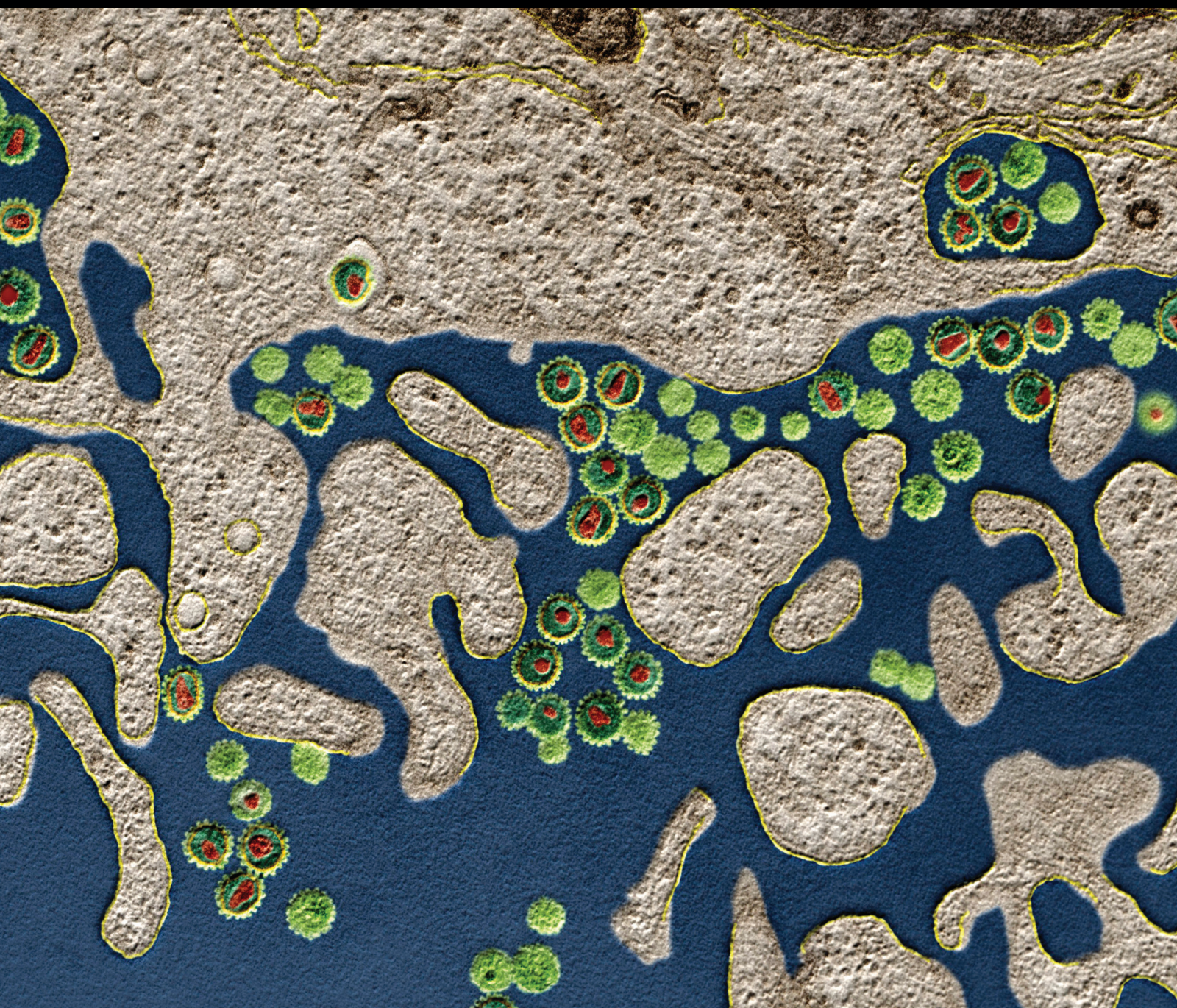
Journal of Immunology Research

Immune System and Chronic Diseases 2018

Lead Guest Editor: Margarete D. Bagatini
Guest Editors: Andréia M. Cardoso, Cristina R. Reschke, and Fabiano Carvalho



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Editorial Board

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Editorial
Immune System and Chronic Diseases 2018

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The immune system has a central role in many processes involving chronic diseases. The recognition of altered immune system function in many chronic disease states has proven to be a pivotal advance in biomedical research over the past decade. For many metabolic disorders, this altered immune activity has been characterized as inflammation. However, accumulating evidence challenges this assumption and suggests that the immune system may be mounting adaptive responses to chronic stressors. Immunological research involving cancer, rheumatoid arthritis, inflammatory bowel disease, asthma, multiple sclerosis diabetes, heart diseases, and others has not only enabled the understanding of the mechanisms that underlie these diseases but also suggested new therapies that may impact positively on patients minimizing morbidity and mortality. This special issue is aimed at presenting and discussing the advancement of research and innovative therapies involving chronic diseases, which have a high impact on society.

Evidences show that purinergic signaling is involved in processes associated with the immune system in health and disease, including noncommunicable, neurological, and degenerative diseases. These diseases strike from children to elderly and are generally characterized by progressive deterioration of cells, eventually leading to tissue or organ degeneration. These pathological conditions can be associated with disturbance in the signaling mediated by nucleotides and nucleosides of adenine, in expression or activity of extracellular ectonucleotidases, and in activation of P2X and P2Y receptors. Among these diseases, the best known are atherosclerosis, hypertension, cancer, epilepsy, Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS). The currently available treatments present limited effectiveness and are mostly palliative. M. D. Bagatini and collaborators reviewed the role of purinergic signaling highlighting the ectonucleotidases E-NTPDase, E-NPP, E-5-nucleotidase, and adenosine deaminase in noncommunicable, neurological, and degenerative diseases associated with the cardiovascular and central nervous systems and cancer.

Considering the involvement of the immune system in cancer, Y. Matsukiyo and colleagues conducted a clinical study that evaluated host immunity in patients with cirrhosis receiving partial splenic embolization (PSE) for thrombocytopenia. Restoration of the balance between T lymphocyte subsets and Th1/Th2 cytokines followed by an improvement of antitumor immunity has been reported after hepatosplenectomy in patients with liver cirrhosis and hepatocellular carcinoma. In fact, after PSE, an increase in counts for platelets, neutrophils, lymphocytes, and monocytes was verified. In parallel, Th1 and Th2 as well as TNF-alpha, TNF receptor I, and soluble Fas showed a significant increase at 4 weeks after PSE. These evidences found by Y. Matsukiyo and collaborators indicate that PSE can recover leukopenia and thrombocytopenia in patients with cirrhosis and thrombocytopenia.

In this line, H. G. Dursun and colleagues studied the association of cytotoxic T lymphocyte antigen-4 gene polymorphisms with psoriasis vulgaris in Turkish population

and determined whether CTLA-4 gene polymorphisms are associated with the development and/or clinical features of psoriasis vulgaris. Psoriasis is a common, chronic, and autoimmune skin disease in which dysregulation of immune cells, particularly T cells, is thought to play an important role in the pathogenesis. Cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed only on activated T cells is an immunoregulatory molecule and plays a role in the pathogenesis of autoimmune disorders. In the same disease, S. Cohen et al. found high expression levels of the Gi protein-associated A3 adenosine receptor (A3AR) and explored its role in mediating the anti-inflammatory effect of piclidenoson, a selective agonist at the A3AR.

Inflammation is the central driving force in much of these chronic degenerative diseases. Noncommunicable inflammatory diseases affect nearly all organ systems of the body including the skin, endocrine glands, gut, lungs, kidneys, and musculoskeletal and cardiovascular system. S. Schröder et al. investigated the modulation of inflammatory reactions after low-dose radiotherapy (LD-RT) on the basis of endothelial cells (EC) and showed that LD-RT, also using very low radiation doses, has a clear immunomodulatory effect on EC as major participants and regulators of inflammation.

Rheumatoid arthritis (RA) is an often debilitating autoinflammatory disease, and patients with this pathology commonly present psychological symptoms. From this, M. Figueiredo-Braga and colleagues carried out a cohort study in RA patients with the purpose of verifying the influence of the levels of cytokines and their inhibitors on psychological symptoms more frequently described. Depression, anxiety, sleep, fatigue, and relationship status were the psychological symptoms investigated and associated with levels of cytokines. They have verified that IL-10 and IL-6 are likely to be involved with depressive symptoms. IL-10 concentration was associated with depression, and tocilizumab decreased depressive symptoms in the RA patients. In parallel, IL-6 was also associated with depressive symptoms in patients with primary depression. These findings contributed significantly to understanding possible mechanisms that trigger psychological symptoms commonly found in RA. In addition, the study highlights that cytokine levels have a strong impact on depressive symptoms.

R. A. Iseme et al. studied the association between autoantibodies and qualitative ultrasound index of bone in an elderly sample without clinical autoimmune disease. Bone loss is characteristic of the ageing process and a common complication of many autoimmune diseases. Research has highlighted a potential role of autoantibodies in pathologic bone loss. In this line, F. M. Perrotta et al. investigated the serum levels of sclerostin in patients with ankylosing spondylitis (AS) as a possible biomarker and for investigating any correlations with radiographic damage, disease activity, and function, considering that several molecules are involved in the pathogenesis of a new bone formation.

M. R. Barros Jr. et al. reviewed the HPV immune evasion mechanisms involving Toll-like receptors (TLRs) and cytokines and discussed the existing and potential immunotherapeutic TLR- and cytokine-related tools. The authors revealed that HPV-related tumors require a great immune suppressor status for cancer development with increased activities of Treg, CTLA-4, and PD-1 and the suppression of APC and NK cells and that the therapies should consider the tumor-related evasion mechanisms. H.W. Grievink and M. Moerland speculated that sample aging induces an inhibitory pathway downstream from Toll-like receptor 4 in monocytes. These results underline the importance of quick sample handling when investigating innate immune responses in whole blood, especially for monocyte responses. S. Wang et al. evaluated the protective effects of sublancin on immunosuppression in cyclophosphamide-treated mice and suggested that sublancin plays a crucial role in the protection against immunosuppression in cyclophosphamide-treated mice and could be a potential candidate for immune therapy regimens.

A. Puccetti et al. identified specific miRNA signatures associated with Behçet's disease (BD) patients with active disease and concluded that the combined analysis of deregulated miRNAs and BD transcriptome sheds light on some epigenetic aspects of BD identifying specific miRNAs, which may represent promising candidates as biomarkers and/or for the design of novel therapeutic strategies in BD. R. A. G. Khammissa et al. reviewed the clinical and histopathological aspects of adverse immunologically mediated oral mucosal reactions to systemic medication, which seems to be occurring in genetic susceptible patients.

Bronchial asthma is an important cause of chronic morbidity affecting children and adults worldwide. In this pathology, eosinophil infiltration releases a number of cationic proteins, including the major basic protein (MBP) and the eosinophil cationic protein (ECP), which can lead to the damage of the bronchial epithelial cells. To understand the role of cationic proteins in bronchial epithelial cells, Y.-N. Wang and colleagues conducted an experimental investigation to find out whether poly-L-arginine (PLA), a synthetic cationic protein, induces apoptosis in the human airway epithelial cells (NCI-H292) as well as cellular targets involved in this effect. In fact, PLA triggered apoptosis in NCI-H292 cells and altered the signaling pathways ERK1/2 and Bcl2/Bax. These evidences point to the inhibition of cationic protein as a potential target of anti-injury or antiremodeling in asthmatics.

Conflicts of Interest

The authors declare to have no conflict of interest in this special issue.

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Review Article

The Impact of Purinergic System Enzymes on Noncommunicable, Neurological, and Degenerative Diseases

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Evidences show that purinergic signaling is involved in processes associated with health and disease, including noncommunicable, neurological, and degenerative diseases. These diseases strike from children to elderly and are generally characterized by progressive deterioration of cells, eventually leading to tissue or organ degeneration. These pathological conditions can be associated with disturbance in the signaling mediated by nucleotides and nucleosides of adenine, in expression or activity of extracellular ectonucleotidases and in activation of P2X and P2Y receptors. Among the best known of these diseases are atherosclerosis, hypertension, cancer, epilepsy, Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS). The currently available treatments present limited effectiveness and are mostly palliative. This review aims to present the role of purinergic signaling highlighting the ectonucleotidases E-NTPDase, E-NPP, E-5′-nucleotidase, and adenosine deaminase in noncommunicable, neurological, and degenerative diseases associated with the cardiovascular and central nervous systems and cancer. In conclusion, changes in the activity of ectonucleotidases were verified in all reviewed diseases. Although the role of ectonucleotidases still remains to be further investigated, evidences reviewed here can contribute to a better understanding of the molecular mechanisms of highly complex diseases, which majorly impact on patients' quality of life.

1. Introduction

Noncommunicable, neurological, and degenerative diseases are characterized by cell loss, ultimately leading to deterioration in quality or function of tissues or organs and possible failure of vital organs [1]. Although the etiology and pathogenesis of these diseases remain unclear, recent advances indicate that the processes of organ deterioration share common core features, including cell injury and dysfunction that contribute to functional and morphological impairment of cells. Despite considerable progress in understanding the molecular mechanisms of these diseases, current therapeutic options are limited, and no effective pharmacological treatment has emerged to date. Elucidation of common and unique mechanisms responsible for the deterioration present in these pathologies may facilitate the identification and development of effective targets and therapies [2]. Furthermore, the search for specific (bio) markers for each human condition—physiological and pathological—is becoming critical.

Elements of the purinergic signaling system are involved in many processes in health and disease conditions [3].

Therefore, a complete understanding of purinergic system could potentially unveil possible markers or relevant pathways for pathological processes, mainly related to human degeneration. Briefly, the purinergic system consists of three main components: (i) the extracellular nucleotides and nucleosides, which mediate signaling; (ii) the receptors through which these nucleotides and nucleosides exert their effects; (iii) and the ectoenzymes, responsible for the control of extracellular levels of these molecules [4]. The control of the levels of extracellular nucleotides adenine and adenosine and the consequent signaling by purinergic receptors induced by them is critical in maintaining the physiological processes [5]. This control is performed by ectonucleotidases, which are enzymes anchored to the cell surface or located in the interstitial medium (soluble form) [6].

2. Purinergic System

Purines' extracellular role was first demonstrated in 1929 by Drury and Szent-Györgyi [7], which described its actions in mammary hearts [8–10]. Although, only in 1970, Burnstock proposed the term "purinergic" and presented his hypothesis about ATP as an independent neurotransmitter released from nonadrenergic noncholinergic neurons in the intestines, bladder, and gut [11, 12]. Two years later, Burnstock described adenosine triphosphate (ATP) as an extracellular signaling molecule and its effects [13]. However, the purinergic system and ATP had an arduous path to be accepted by the scientific community. Only in 2006, ATP was finally recognized as a cotransmitter in both the peripheral and central nervous systems (CNS) [9, 10, 14], and the purinergic signaling was recognized as a system involved in many nonneuronal and neuronal mechanisms [12].

ATP is the most versatile nucleotide and the primary energy source for cellular functions. Hundreds of reactions in the cell, from metabolic transformations to signaling events, are coupled to the hydrolysis of ATP [15]. Intracellularly, ATP is stored at very high levels (from 5 to 10 mmol/l), which can quickly be degraded by ubiquitous extracellular nucleotidases after connecting to specific receptors under physiological conditions. In fact, extracellular ATP has an extremely short half-life before it is degraded to adenosine—milliseconds to seconds. This rapid breakdown results in the activation of a multiplicity of receptor subtypes, which can mediate physiological processes such as proliferation, differentiation, migration, and cell death [16]. On the other hand, the excess of ATP in the brain extracellular space can induce neurotoxicity [17].

ATP stores energy by losing a phosphate group and forming ADP. It has been shown that the ADP molecule can have an important role in platelet aggregation (platelet granules contain high concentrations of ADP), blood vessel tone, cardioprotection, and vascular wall integrity [18].

The critical functions of ATP and its subsequent hydrolysis are initiated upon binding to purinergic receptors, such as P2 nucleotide and P1 adenosine receptors [19]. Abbracchio and Burnstock divided P2 receptors into two families: P2X family of ligand-gated ion channel receptors and the P2Y family of G protein-coupled receptors, based on their molecular structure, induced mechanism of action, and the sequence analysis of cloned P2 receptors [20].

Currently, thirteen human P2X receptor subtypes can be distinguished: 6 homomeric (P2X1, P2X2, P2X3, P2X4, P2X5, and P2X7) and 7 heteromeric (P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6 [21], and P2X4/7) [22]. P2X receptors are nonselective ligand-gated ion channels that mediate sodium influx, potassium efflux, and at some extent calcium influx, leading to cell membrane depolarization [23]. The P2X2/3 receptors are located in the nodose ganglia [24], P2X4/6/7 in the CNS [25, 26], P2X1/5 in the blood vessels, and P2X2/6 receptors are mainly located in the brain stem [24, 27, 28]. P2X receptors have important functions in the central and peripheral nervous systems, such as slow neuromodulatory function, rapid synaptic transmission, neurotransmitter release, and the generation of pain signals. Furthermore, these receptors have pathophysiological role in injury, inflammation, anxiety, dementia, epilepsy [26], and neurodegenerative disorders such as Alzheimer's and Huntington's diseases [29–32].

P2Y receptors are G protein-coupled receptors, virtually present in all cells, and mainly activated by adenine and uridine nucleotides. There are eight subtypes of P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14), which are ubiquitously expressed in body, including the CNS [32]. They activate intracellular signaling cascades to regulate a variety of cellular and peripheral pathophysiological processes, including inflammation, ischemia, and pain [32, 33]. In the brain, P2Y receptors exert important roles in neurotransmission, glia cell communication, and neurogenesis due to their localization on neurons, oligodendrocytes, microglia, and astrocytes [13, 34–36].

After release and binding to specific receptors, ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases [4]. Ectonucleotidase families include the ectonucleoside triphosphate diphosphohydrolases (E-NTPDase/CD39/NTPDase 1), ectonucleotide pyrophosphatase/phosphodiesterases (E-NPP), and ecto-5′-nucleotidase (E-5′-nucleotidase/CD73). E-NTPDases and E-NPPs hydrolyze ATP and ADP to adenosine monophosphate that is further hydrolyzed by E-5′-nucleotidase to adenosine [6, 37, 38].

Since 1929, adenosine has been recognized as a biologically significant molecule, responsible for regulating multiple systems including cardiac conduction, arterial pressure, and intestinal motility [7]. Adenosine is produced both intracellularly and extracellularly through enzymatic degradation of adenine nucleotides [39, 40]. The maintenance of its levels in the extracellular fluids is the result of the balance between its production and consumption [19]. Indeed, adenosine synthesis is controlled by ectonucleotidases located on the cytoplasmatic membrane, and its concentrations vary according to physiological and pathological stimuli such as hypoxia and inflammation [41, 42]. Extracellular adenosine is involved in many cytoprotective functions of the body, including conditioning the heart against ischemia, counteracting the damaging effects of excitotoxicity and seizure activity in the brain, and suppressing excessive immune and inflammatory responses. Finally, extracellular adenosine

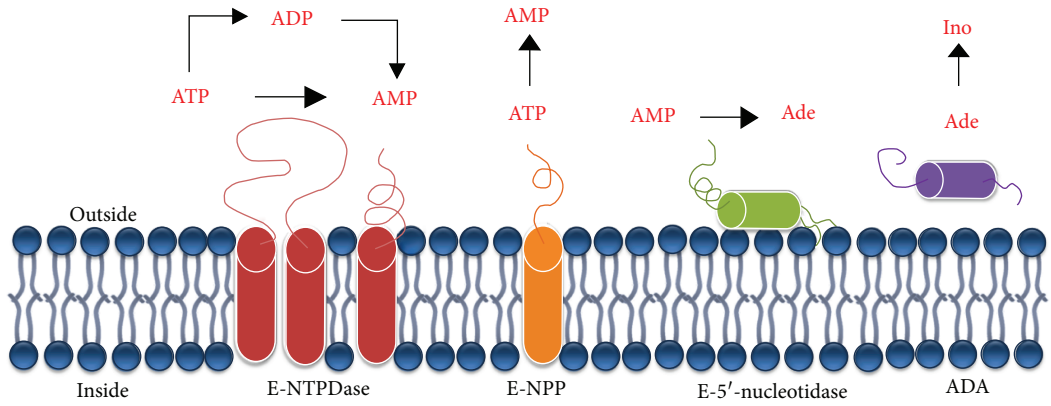

Figure 1: Cell membrane-anchored ectonucleotidases and their respective hydrolysis reactions.

can be deaminated to inosine by adenosine deaminase (ADA) [43].

Critically, the understanding of the physiological and pharmacological roles of adenosine was greatly facilitated by localizing the different P1 receptor subtypes in target tissues and identifying its four subtypes—A1, A2A, A2B, and A3 receptors [44–46]. These are G protein-coupled receptors, which present inhibitory action upon adenosine binding [20]. P1 receptors are widely located in the brain and in peripheral tissues, such as the heart, kidney, and adrenals of different species [45, 46].

After the binding to receptors, the nucleotides and nucleosides are rapidly degraded by specific hydrolase enzymes, which occurs via different ways [47]. The E-NTPDase catalyzes ATP and ADP hydrolyses, which culminates into AMP formation. E-5′-nucleotidase is responsible for AMP degradation leading to adenosine formation. Alkaline phosphatase removes inorganic phosphate (Pi) of a broad range of substrates, including nucleotides (ATP, ADP, and AMP) producing adenosine (Figure 1) [48, 49]. The producing adenosine is converted into inosine by adenosine deaminase (ADA) [38].

The E-NTPDase family is very efficient in controlling the bioavailability of ATP [49]. This family consists of eight members, E-NTPDases 1–8, which differ in substrate specificity, tissue distribution, and cellular localization [50]. Members 1, 2, 3, and 8 are the principal enzymes responsible for the hydrolysis of tri- and diphosphate nucleotides on the cell surface under physiological conditions. E-NTPDases 4, 5, 6, and 7 are associated with intracellular organelles [50]. These ectoenzymes are anchored to the plasma membrane via hydrophobic domains with the active site facing the extracellular medium [5, 38, 48].

The E-5′-nucleotidase family is comprised of seven isolated and characterized isoforms with different nomenclature, depending on the subcellular localization. Five isoforms are cytosolic, one is located in the mitochondrial matrix, and one is related to the outer plasma membrane [47]. This enzyme generates extracellular adenosine from AMP, as previously mentioned, and its activity is the rate-limiting step of adenosine formation from adenine nucleotides in most tissues [49, 51].

The E-NPPs are transmembrane glycoprotein type II enzymes, which are able to catalyze many different reactions: 3′-5′-cyclic adenosine monophosphate (5′-cAMP) to AMP; ATP to AMP and inorganic diphosphate (PPi); AMP to ADP and inorganic phosphate (Pi); nicotinamide adenine dinucleotide oxidized (NAD^+) to AMP and mononucleotide nicotinamide [6]. Although this family of enzymes is composed by 7 members, only E-NPPs 1, 2, and 3 are able to hydrolyze nucleotides [5].

ADA activity has been found altered in various pathological conditions including acquired immunodeficiency syndrome (AIDS), anemia, lymphomas, tuberculosis, and leukemia [47]. In humans, different ADA isoforms have been identified: adenosine deaminase-1 (ADA-1), adenosine deaminase-2 (ADA-2), and adenosine deaminase-3 (ADA-3) [52]. ADA-1 plays a metabolic role not only as a key cytosolic enzyme in the purine pathway but also as an ectoenzyme by regulating extracellular adenosine levels. In contrast, ADA-2 is specifically designed to act in the extracellular environment according to its presence in the serum [53]. Thus, ADA can be considered as a multifunctional protein, playing several roles and importantly regulating biological systems, which consequently impacts on human health [53].

3. Noncommunicable, Neurological, and Degenerative Diseases

According to the World Health Organization (WHO), chronic disorders account for 38 million deaths each year, more than 80% of all deaths worldwide. Cardiovascular diseases cause most of the deaths (17.5 million per annum), followed by cancers (8.2 million), respiratory diseases (4 million), and diabetes (1.5 million) [54, 55]. In the last two decades, this profile of morbidity and mortality causes around the world has significantly been changed, and, increasingly, chronic neurological and degenerative diseases are becoming more prominent [54].

Among the better-known chronic diseases are the noncommunicable, such as atherosclerosis, hypertension, diabetes, and cancer; the neurological, such as epilepsy; and the neurodegenerative, such as Alzheimer's disease (AD),

Table 1: Ectonucleotidases (E-NTPDase, E-5′-nucleotidase, and ADA) and purinergic receptors in experimental models and patients with atherosclerosis.

Sample	E-NTPDase	E-5′-nucleotidase	Adenosine deaminase	Involved receptors	Reference
Endothelial cells and thoracic aorta from mouse model of atherosclerosis	↓ enzymatic activity and expression	—	—	—	[72, 84]
ApoE-deficient mice	—	↓ enzymatic activity and expression	—	—	[88]
Platelets and plasma from patients	↓ enzymatic activity	↑ enzymatic activity	—	—	[90]
Plasma of experimental model	—	—	↑ enzymatic activity	A1 upregulation	[93]
Atherosclerotic vessel wall from aortoiliac bifurcation of Apoliprotein E- and LDL-deficient animals	—	—	↓ enzymatic activity	—	[92]

Parkinson's disease (PD), and multiple sclerosis (MS). The study and control of these diseases are exceedingly difficult due to their multiplicity and diversity, for instance, the interconnection network of risk and protective factors, diverse onsets followed by multistep pathogenesis, and, in some cases, multifocal localization [56]. A common feature among many of the neurological, neurodegenerative, and several aspects of cardiovascular diseases may be a component of degeneration. It means that at some stage occurs loss of cells, subcellular function, or tissue elements or function [57]. In fact, these diseases may also share other pathological features or events, such as evidence of membrane damage, oxidative stress, mitochondrial dysfunction, and upregulation of autophagy [58].

Once purinergic signaling is involved in virtually all body functions, the roles of purines might be altered in pathological states in different body systems [59]. Indeed, purinergic mechanisms and specific receptor subtypes have been shown to be involved in several pathologies including brain trauma and ischemia, neurodegenerative diseases involving neuroimmune and neuroinflammatory reactions [60], diabetes [61], vascular diseases including atherosclerosis [62, 63], and hypertension [9, 10]. Importantly, studies have shown the potential of purinergic mechanisms as therapeutic targets for the treatment of neurological [36] and degenerative disorders [9, 64, 65]. Moreover, the currently used strategies in searching for novel purinergic targeting drugs include the development of (i) selective agonist and antagonist ligands for the P2X and P2Y receptors, (ii) inhibitors of extracellular catabolism of purines, and (iii) modulators of nucleotide and nucleoside transport [66]. Adenosine signaling manipulation may also have therapeutic potential in neurodegenerative diseases such as AD, PD, and Huntington's diseases and in neurological and psychiatric disorders such as epilepsy [67], schizophrenia, and autism [68].

Taking that into account, the next section provides an overview of recent findings regarding the role of purinergic system enzymes in the pathophysiology of noncommunicable, neurological, and degenerative diseases. Considerable attention has been directed towards diseases related to the cardiovascular system (atherosclerosis and hypertension), cancer, and central nervous system (epilepsy, Alzheimer's disease, Parkinson's disease, and multiple sclerosis).

3.1. Atherosclerosis. Atherosclerosis is popularly defined as an artery wall thickening triggered by accumulation of foam cells and proliferation of intimal smooth muscle cell, which results in a fibrofatty plaque. Atherosclerotic disease still is the worldwide leading cause of cardiovascular complications and death [69]. The drivers for atherosclerotic plaque development include fluid shear forces, lipid milieu, cells of the vascular wall, and cells recruited from the circulation. However, one of the central events that results in the atherosclerosis development is the disturbance in blood flow in human arteries. In addition, some cells, such as endothelial cells, platelets, monocytes, and T cells, can be involved and/or activated in response to changes in blood flow [70].

Arteries presenting constant flow are further resistant to plaque formation when compared to arteries presenting disturbance in blood flow. Taking this into account, it is important to point out that the aspects that control the blood flow, such as genes, microRNAs, and epigenomic processes, initiate responses that can result in activated or quiescent endothelial phenotypes and, in turn, susceptibility to or protection from atherosclerosis [70–72]. Corroborating with this, Nam and collaborators demonstrated that disturbed flow directly causes atherosclerosis within 14 days, under hyperlipidemic conditions, after partial carotid ligation surgery [71].

It is well stablished that E-NTPDase 1 is the major ectonucleotidase expressed in blood vessels. Several studies have been carried out in order to elucidate the role of this enzyme in controlling the blood flow, atherosclerosis development, and inflammation related to vascular disorders [70, 73] (Table 1). It has been widely shown that E-NTPDase 1 expressed in both endothelial and smooth muscle cells, along with autonomic nervous system, can control the contraction of arteries, which directly influences the regulation of blood circulation [49, 74–77]. Moreover, E-NTPDase 1 plays a critical role limiting the activation of some P2 receptors, thus, preventing its desensitization in endothelial and smooth muscle cells, consequently, preventing excessive artery contraction [76–79]. This fact can be explained because ATP, released from sympathetic nerves, along with norepinephrine (NE) and neuropeptide Y, can activate P2X receptors at postjunctional membranes evoking vascular smooth muscle

contraction, which generally potentiate the effects of NE at α1-adrenoceptors [51, 80]. Accordingly, Cardoso et al. have shown that E-NTPDase 1 expression and activity have the ability to control the effects of sympathetic nervous system in the vasculature [81].

Regarding to the vascular injury related to atherogenesis, it has been shown that E-NTPDase 1 naturally results in protection from the action of nucleotides released from the injured cell in vasculature or activated platelets by eliminating these prothrombotic and proinflammatory stimuli, that is, by metabolizing extracellular ATP and ADP. In a recent work, Kanthi et al. have evaluated the role of E-NTPDase 1 in the development of atherogenesis, in an apolipoprotein E-deletion mouse model of atherosclerosis (ApoE-deficient) [70]. ApoE-deficient group, fed with a high-fat diet, showed evidently augmented and plaque formation along with platelet activation that was verified by some circulating markers. In this work, the authors also showed that in regions of stable flow, E-NTPDase 1 was markedly present in ApoE-deficient mice. In contrast, E-NTPDase 1 was reduced in atheroprone as subject to disturbed flow. Moreover, in this same work, it has been demonstrated that the deregulated flow triggered by a partial carotid artery ligation quickly suppressed endothelial E-NTPDase 1 expression. Furthermore, unidirectional laminar shear stress induced atheroprotective E-NTPDase 1 expression in human endothelial cells. In these conditions, the vascular transcription factor involved in the E-NTPDase 1 is the Krüppel-like factor 2 (KLF2), which can bind near the transcriptional start site of E-NTPDase 1.

Mercier and collaborators have shown that ApoE knockout mice displayed decreased E-NTPDase 1 expression and activity in the thoracic aorta, which correlates with reduced vascular reactivity and presence of atherosclerotic plaques in aortic roots and arches. The authors have speculated that the accumulation of ATP and ADP in ApoE knockout mice may cause desensitization of P2 receptors, which in turn may contribute to decreased blood flow and may predispose an individual to atheroma formation [82]. In a carotid artery wire injury model, E-NTPDase 1 knockout mice showed decreased migration of vascular smooth muscle cells and reduced neointimal formation, indicating that CD39 contributes to a harmful neointima formation [83]. Taken together, these data establish that the key regulator of atherosclesosis development driven by shear stress is the E-NTPDase 1 enzyme. Kanthi et al. have unveiled what is now believed to be a previously unrecognized role for E-NTPDase 1 as an endogenous regulator of endovascular purine levels, serving as a modulator of crucial cellular drivers of atherogenesis [70]. Emphasizing the role of E-NTPDase 1 in providing anti-inflammatory and antithrombotic mediator adenosine, it has been shown that therapeutic strategies targeting CD39 offer promising opportunities in the management of vascular thromboinflammatory diseases [73].

CD73 also has been reported playing a key role in the atherogenic process by driving purinergic signaling [84, 85]. In a work developed by Buchheiser et al., it was demonstrated that the inactivation of CD73 promotes atherogenesis in apolipoprotein E-deficient mice [86]. The ablation of CD73 in ApoE knockout animals triggered an augmentation in atheroma formation, which was probably generated by the reduced inhibitory control exerted by adenosine on immune cells, which are dynamically related to the atherogenic process [86]. The role of CD73 in protection against atherosclerosis was also observed after injury of carotid arteries. This condition results in augmented expression of vascular cell adhesion molecule- (VCAM-) 1 and increased nuclear factor-kappa B activity, which triggers the formation of neointimal plaque and continuous infiltration of macrophage in CD73 knockout animals when compared to control groups [87].

Jalkanen and collaborators have evaluated 226 patients with stable peripheral artery disease admitted for nonurgent invasive imaging and treatment. They verified that the progression of atherosclerosis is associated with low E-NTPDase 1 activity and high CD73 [88]. Same authors also found high levels of ATP and ADP in the plasma of those patients, suggesting that low E-NTPDase 1 activity is associated with ATP- and ADP-induced platelet aggregation and trombus formation [88].

Since adenosine is the main molecule that presents antithrombotic effects, ADA also has a key role in atherogenesis process. This enzyme has been suggested as an inflammatory marker [89], and its high expression and activity were associated with the risk of atherosclerosis development [89, 90]. Kalvegren et al. have verified changes in metabolism of extracellular nucleotides in the atherosclerotic vessel wall from aortoiliac bifurcation of apoliprotein E- and LDL-deficient animals (ApoE/LDLr (−/−)). They observed that the ADA activity was decreased as well as the levels of adenosine in ApoE/LDLr (−/−) mice when compared to littermate control, reinforcing the idea that low ADA activity is a predictor of atherogenesis. Another mechanism that can contribute to the development of atherosclerosis is that high levels of ADA can increase the release of reactive oxygen species from neutrophils, through a downregulation of the inhibitory adenosine/cAMP system and an enhanced activation of A1 receptors. This sustained neutrophil activation could also contribute to inflammatory disorders and atherogenesis [91].

Genetic studies have been performed regarding the relationship between ADA and atherosclerosis, and two main points can be highlighted: (1) single-nucleotide polymorphisms of the RNA-specific gene of ADA are associated with metabolic disorders in general and atherosclerosis [92] and (2) high ADA gene expression is related to the augmentation of cathepsin S mRNA, which encodes a cysteine protease associated with angiogenesis and atherosclerosis [93].

Taking the above information into account, it is possible to conclude that a high expression and activity of E-NTPDase 1 and CD73, in order to generate adenosine, and a low expression and activity of ADA, in order to keep the levels and let the adenosine molecule exerts its antithrombotic effects, may exert a positive effect in preventing atherosclerosis.

3.2. Hypertension. Hypertension is a highly prevalent disease, which is estimated to affect 26% of the worldwide adult population [94, 95]. Hypertension is defined by a systolic

blood pressure (BP) of 140 mmHg or higher, a diastolic BP of 90 mmHg or higher, or currently using BP-lowering drugs [95]. The disease represents a major risk factor for the development of kidney failure, coronary events, cerebrum-vascular disease, heart failure, and peripheral vascular disease [94, 95]. Hypertension is the leading cause of morbidity and mortality worldwide while cigarette-smoking is the major preventable cause of death [96]. Nowadays, most patients require more than one drug to achieve BP target, and mono-therapy would only be sufficient in about 20–30% of patients [95]. This fact highlights the unmet need to unveil other mechanisms that can be associated with hypertension development and maintenance.

Among the events related to hypertension, we can cite platelet activation and aggregation, vasoconstriction, and low-grade inflammatory status. It is well known that the purinergic system enzymes are tightly involved regulating nucleotides that can trigger or protect against platelet aggregation. Moreover, the control of extracellular circulating nucleotides by ectonucleotidases is related to both anti- and proinflammatory status [49]. Recently, Fabbiano and collaborators have demonstrated that regulatory T (TReg) cell-expressing E-NTPDase 1 require an immunosuppression-independent mechanism to counteract renal and possibly cardiac damage during angiotensin II- (AngII-) dependent hypertension [97]. Tissue-resident neutrophils suffer apoptosis by ATP hydrolysis of E-NTPDase 1. The same group also stated that genetic alterations in the number of TReg and TH cells has an influence on tissue-resident neutrophil number, cardiomyocyte hypertrophy, cardiorenal fibrosis, and elevation of arterial pressure during AngII-induced hypertension [97]. Thus, TReg cell-expressing E-NTPDase 1 can protect against hypertension-driven fibrosis in tissue.

In pulmonary arterial hypertension (PAH), two independent studies have been performed with different outcomes. Using human samples, *in vitro* strategies, and a rat model, Helenius et al. have demonstrated that the suppression of E-NTPDase 1 is linked to the pathogenesis of PAH [98]. Furthermore, they stated that the accumulation of extracellular ATP and ADP is strictly linked to vascular dysfunction and remodeling in PAH and can modulate the disease course in multiple levels. On the other hand, Visovatti et al. have observed that microparticles from platelets and endothelial cells of patients with PAH display increased E-NTPDase 1 activity and expression [99]. Therefore, more studies are crucial to elucidate whether high or low levels of E-NTPDase 1 can be related to PAH development.

Taking advantage of a preeclampsia mouse model, McRae et al. have evaluated the impact of E-NTPDase 1 overexpression. In this study, the authors injected Th1-polarized cells on pregnancy days 10 and 12 of wild-type and E-NTPDase 1 transgenic mice and measured the systolic blood pressure (SBP) until pregnancy day 15, when mice were sacrificed. Following transfer of Th1-polarized cells, SBP of E-NTPDase 1 transgenic mice remained unchanged, without evidence of renal lesions, while an increase was observed in pregnant wild-type mice. Thus, the authors have concluded that E-NTPDase 1 overexpression can be protective in a mouse model of preeclampsia [100].

Sympathetic system has a crucial link with hypertension development by controlling vascular tonus. E-NTPDase 2 is associated with the adventitia of muscularized vessels, microvascular pericytes, and other cell populations in the subendocardial space in the heart [75]. Later, Rücker et al. have shown that E-NTPDase 2 is the most expressed ectonucleotidase in synaptosomes prepared from rat heart left ventricles, indicating that this enzyme may be essential for modulating ATP and NE responses on heart fibers [101]. At the same time, the presence of E-NTPDase 2 in the vascular murine adventitial cells [75] also suggests that this enzyme plays a role in hydrolyzing the ATP released from sympathetic nerves and, thus, can help to control vascular tone and hypertension development.

E-5′-nucleotidase is also highly expressed in the left ventricle, and its activity seems to be important for the control of the nucleotide/nucleoside ratio in the vicinity of nerve endings in the heart [101]. Moreover, in human coronary arteries, sympathetic nerves are one of the sources of adenine nucleotides and coronary vasodilation was found to be associated with the endothelial expression of E-5′-nucleotidase. Inhibition of E-5′-nucleotidase and the usage of P1 antagonists have been associated with a marked reduction of the relaxation of coronary arteries [102]. The same occurs in mesenteric arteries [103] and other vascular beds [51]. Data from Sousa and colleagues have revealed that the increase in the sympathetic tonus in spontaneously hypertensive rats can be associated to a higher NE/ATP release ratio from sympathetic nerves and to deficits in the endogenous inhibitory tonus mediated by prejunctional adenosine A1 receptors [103]. This fact reinforces the important role of E-NTPDase 1, E-NTPDase 2, and CD73 in producing adenosine and preventing hypertension development caused by augmented sympathetic tonus. Indeed, adenosine produced by E-5′-nucleotidase activates P1 receptors, which can cause hyperpolarization and relaxation of the underlying vascular smooth muscle cells [102].

Increased E-NTPDase and CD73 activities have been observed in both animal models of hypertension, in human studies, and in platelets and lymphocytes [81, 104–107]. These specific platelet and lymphocyte responses can be understood as a mechanism to ameliorate hypertension through the elevation of adenosine levels, by combined actions of E-NTPDase and CD73.

Taken together, these results indicate that E-NTPDase 1 and CD73 expressed in lymphocytes, TReg cells, endothelial cells, and platelets constitute a protective barrier against hypertension-driven tissue fibrosis. In addition, the results suggest new therapeutic avenues to prevent hypertension and hypertension-linked pathologies.

Changes in ADA expression and activity are also related to hypertension development and maintenance. Human studies have shown increase in ADA activity and expression in response to hypertension and hypertension-associated pathologies, such as metabolic syndrome [108]. A genetic study with hypertensive patients showed that a common polymorphism (C34T) of the ADA gene (isoform 1) is strongly correlated with essential hypertension [109]. ADA

Table 2: Ectonucleotidases (E-NTPDase, E-5′-nucleotidase, and ADA) and purinergic receptors in experimental models and patients with hypertension.

Sample	E-NTPDase	E-5′-nucleotidase	Adenosine deaminase	Involved receptors	Reference
Treg cells from angiotensin II-dependent hypertension	↑ expression CD39 ↑ enzymatic activity to ATP and ADP	—	—	—	[99]
Pulmonary arterial hypertension	↑ expression CD39 ↑ enzymatic activity to ATP and ADP	—	—	—	[100, 101]
Preeclampsia	↑ expression CD39 can protect against preeclampsia	—	—	—	[102]
Synaptosomes from rat heart	↑ expression of NTPDase 2	↑ expression			[77, 103]
Human coronary arteries	—	↑ expression	—	P1 upregulation	[104]
Platelets and lymphocytes of hypertensive human	↑ expression ↑ enzymatic activity	↑ enzymatic activity	↑ enzymatic activity	—	[109–111]
Animal membrane fractions of renal tissue	—	—	↓ enzymatic activity and expression	A2A downregulation A3 upregulation	[113]
Platelet of pregnant hypertensive woman	—	—	↑ enzymatic activity	—	[114, 115]
Placenta of pregnant hypertensive woman	—	↑ expression ↑ enzymatic activity	—	—	[116]

activity is also increased in both platelets and lymphocytes in an animal model of hypertension induced by L-NAME administration [81, 104–106].

Moreover, Tofovic et al. have suggested that the inhibition of ADA may provide beneficial effects in old hypertensive animals, and an inhibitor of this enzyme could be designed and used to offer cardiovascular protection in hypertension [110]. Franco and colleagues have analyzed the activity of nucleotidases and ADA in cytosolic and membrane fractions of renal tissue, in an angiotensin-II model of hypertension [111]. They observed a decrease in the membrane ADA activity and expression, in AngII-treated rats. Furthermore, despite the adenosine elevation, A1 and A2B receptor protein expression did not change; in contrast, a downregulation was observed in A2A receptors and an upregulation in A3 receptor levels. A similar pattern was found in the cortex and in the medulla—the expression of A3 receptor decreased in both segments. These results suggest that the elevation of renal tissue and interstitial adenosine contributes to the renal vasoconstriction observed in the AngII-induced hypertension. This can be either mediated by a decrease in the activity and expression of ADA, increased production of adenosine, or an imbalance in adenosine receptors [111].

Original studies have shown increased platelet aggregation as a result of high ADA activity and expression and lack of adenosine in pregnancy hypertensive [112, 113]. It was indeed suggested that ADA activity as well as platelet aggregation could serve as peripheral markers for the development of therapy for the maintenance of homeostasis and inflammatory processes in hypertension and hypertension-associated pathologies [108, 113]. In contrast, Iriyama et al. have analyzed adenosine metabolism using two different animal models of preeclampsia [114]. They have demonstrated that adenosine levels were high in preeclampsia, and this increase was enough to induce hallmark features of preeclampsia including hypertension, proteinuria, small fetuses, and impaired placental vasculature. This study has also revealed that besides the high levels of adenosine, the receptor A2B was excessively activated, contributing to the development of preeclampsia-related features, although the key finding of this study was that the placental adenosine increase is triggered by the elevation of placental CD73, thereby contributing to preeclampsia [114]. Discrepancies between those two studies can be explained by experimental design differences, for instance, site of analysis (platelets versus placenta). Finally, Iriyama et al. did not evaluate the ADA enzyme. Therefore, more studies are necessary to elucidate whether adenosine causes or protects against preeclampsia and to unveil the actual role of ADA in preeclampsia. This information is summarized in Table 2.

3.3. Cancer. Cancers are considered heterogeneous pathologies that vary by tissue of origin and genomic, proteomic, and metabolic alterations [115]. The inflammation plays an important role in the steps required for cancer metastasis because a wide variety of cytokines and other proinflammatory markers contributes to both the extrinsic and intrinsic pathways of inflammation associated by cancer [116].

The enzymatic chain responsible for ATP hydrolysis and adenosine production, present in all immune and vascular cells, has an important role in the control or promotion

of inflammation in tumors [117]. This is because of an exuberant immune/inflammatory response, not adequately balanced by endogenous mechanisms of homeostatic control that can lead to persistent and abnormal forms of collateral tissue damage [118]. Adenosine can accumulate in the tumor environment and stroma and generate an immunosuppressed microenvironment that favors the development and metastasis of neoplasias [119].

Multiple mechanisms are involved in adenosine effects, which include inhibition of T helper 1 cell (TH1 cell) cytokine production, deregulation of mononuclear phagocyte cell differentiation and maturation, suppression of effector T cells, and generation of an angiogenic and matrix remodelling environment that is suitable for cancer growth [120, 121]. Most of the signaling actions of extracellular adenosine are mediated by G protein-coupled cell surface receptors that are divided into four subtypes: A1, A2A, A2B, and A3 [122].

The extracellular adenosine acts as a local modulator and exerts effects by protecting cells and tissues from an excessive inflammatory response and as favorable to the onset and cancer growth, by favoring angiogenesis and matrix remodeling [122, 123]. When adenosine active A2A, A2B, and A3 receptors, macrophages promotes the release of the anti-inflammatory cytokine like as TNF, IL-6, IL-10, IL-12, nitric oxide (NO) and macrophage inflamatory protein-(MIP)-1α [52, 120].

The A2A receptor activity, a T-cell surface immune checkpoint protein, could lead to the discovery that adenosine in the tumor microenvironment interferes with antitumor immunity, suggesting that antagonism of the A2A could be an effective cancer immunotherapeutic [124].

3.4. Epilepsy. Epilepsy is a debilitating neurological disease, characterized by recurring, spontaneous, unprovoked seizures. Temporal lobe epilepsy (TLE) is the most common form of acquired epilepsy, affecting people from all ages [125]. The World Health Organization (WHO) reports that epilepsy affects approximately 50 million people worldwide, with about a third of patients either resistant to current antiepileptic drugs (AED) or experiencing unacceptable side effects.

Neuroinflammation is increasingly recognized as one of the key players in seizure generation and propagation and in the maintenance of the epileptic phenotype [126]. During pathological conditions with increased neuronal firing or cell death nucleotides, ATP is released into the extracellular space in the brain. Although studies show discrepancies about ATP release during seizures and chronic epileptic state, this is possibly due to ATP rapid hydrolysis, by ectonucleotidases in the extracellular space, making it difficult to directly measure its release [127]. Once released, ATP activates P2 receptors (P2X and P2Y), mediating the release of gliotransmitters, which triggers neuronal hyperexcitability and neuroinflammation. Taking advantage of experimental models of epilepsy, the expression and function of P2X receptors have been well established in research field [36, 128]. However, only recently has been suggested that the P2Y receptor subfamily plays a relevant role in experimental and human epilepsy [67, 129]. Furthermore, while adenosine anticonvulsant properties are well established [130], the contribution of extracellular nucleotides to seizures and epilepsy pathology is an area to be further explored [36]. Therefore, not only the targeting of molecules involved in inflammatory pathways but also the targeting of purinergic signaling or their combination seems relevant approaches for developing novel therapies for epilepsy [36, 126].

3.5. Alzheimer's Disease. Alzheimer's disease (AD), the leading cause of dementia worldwide, is a progressive neurodegenerative disease associated with the deposition of β-amyloid peptide (Aβ) within the brain, along with intracellular neurofibrillary tangles (NFTs) mainly formed by hyperphosphorylated tau protein [131]. The impairment of mitochondrial function and reduction of ATP levels are pathological conditions found in AD, which is closely linked to the decline of cognitive processes [132, 133]. It is well known that in the brain, ATP is secreted by the neurons, glia, and endothelial cells that constitute the blood-brain barrier. Coincident with its release is the secretion of soluble ectonucleotidases which control the effective ATP concentration by breaking it down to adenosine [134]. The involvement of ectonucleotidases on the process of learning and memory in rats has already been described [135–137]. It has also been shown that P2X7 purinergic receptors are upregulated in the brain of patients with AD and in animal models [138, 139]. Interestingly, inhibition of P2X7 in mice, transgenic for mutant human amyloid precursor protein, reduced the number of amyloid plaques in the hippocampus [140] and stimulation of P2X7 receptors on human macrophages and microglia enhanced the degenerative lesions observed in AD [141] (Tables 3 and 4). Furthermore, direct alterations of purine metabolism have been detected in AD by metabolomics, in the ventricular cerebrospinal fluid at postmortem [142], cerebrospinal fluid in living individuals [142–144], and AD brains [145].

Ectonucleotidase activities may be involved in the early events related to memory acquisition and consolidation of an aversively motivated learning task [135, 136]. Previous studies performed in animals subjected to scopolamine model of dementia, which mimics the memory deficit observed in diseases characterized by impairment in cholinergic neurotransmission, such as AD [146–148], have shown a marked reduction in the ATP levels and changes in ectonucleotidase activities (E-NTPDase, E-5′-nucleotidase, and ADA), in the cerebral cortex and hippocampus of rats [149, 150].

Tissue-nonspecific alkaline phosphatase (TNAP) is one of four alkaline phosphatase isozymes that has been described as an ectonucleotidase being able to cleave all forms of adenosine phosphates, influencing purinergic signaling [48]. Preclinical assays tested on more than 100 AD patients have demonstrated that TNAP activity is significantly increased in the hippocampus of AD patients compared with age-related controls [151]. In fact, it has been shown that the extracellular hyperphosphorylated tau protein coming from damaged neurons must be dephosphorylated to become an agonist of a muscarinic receptor

Table 3: Role of purinoreceptors in experimental models and patients with Alzheimer's disease (AD) and Parkinson's disease (PD).

R Sample	P2X7 receptor	A2A receptor	Reference
Transgenic mouse model of AD (brain slices)	Upregulated	—	[169]
Microglia from AD patients Human microglia treated with amyloid-beta peptide *in vitro* Rat hippocampus after amyloid-beta peptide injection	Upregulated	—	[170]
Transgenic mouse model of AD	Inhibition of P2X7R decreased the number of hippocampal amyloid plaques	—	[171]
Human macrophages and microglia preactivated with amyloid-beta peptide	P2X7R stimulation enhanced secretion of proinflammatory cytokines	—	[172, 173]
Rats injected with 6-OHDA	—	Inhibition of A2AR improved motor performance and cognition	[174]
Rats injected with haloperidol (DA antagonist)	—	Inhibition of A2AR reversed locomotor suppression and tremulous jaw movements	[174, 175]

Alzheimer's disease (AD), P2X7 receptors (P2X7R), adenosine A2A receptor (A2AR), 6-hydroxydopamine (6-OHDA), and dopamine (DA).

Table 4: Ectonucleotidases (E-NTPDase, E-5′-nucleotidase, and adenosine deaminase) in experimental models and patients with Alzheimer's disease (AD) and Parkinson's disease (PD).

R Sample	E-NTPDase	E-5′-nucleotidase	Adenosine deaminase	Alkaline phosphatase (TNAP)	Reference
Synaptosomes of rats injected with scopolamine	↓ enzymatic activity to ATP (hippocampus and cortex) ↓ enzymatic activity to ADP (hippocampus)	↓ enzymatic activity (hippocampus)	↓ enzymatic activity (hippocampus)	—	[169]
Synaptosomes of rats injected with scopolamine	↑ enzymatic activity to ATP (hippocampus and cortex) ↑ enzymatic activity to ADP (hippocampus)	↓ enzymatic activity (hippocampus)	↑ enzymatic activity (hippocampus)	—	[170]
Hippocampus and plasma from patients with AD	—	—	—	↑ enzymatic activity	[171]
Plasma from patients with AD	—	—	—	↑ enzymatic activity	[172, 173]
Serum from patients with PD	↑ enzymatic activity to ATP ↑ enzymatic activity to ADP	—	—	—	[174]

Alzheimer's disease (AD) and Parkinson's disease (PD).

[152]. This event has been associated with increased TNAP expression and unbalances of the intracellular calcium homeostasis and phosphorylation levels of intracellular tau [153]. Increased TNAP levels in the plasma of the AD patients have also been reported in literature [151, 154], suggesting that TNAP is a good biomarker of disease progression. On the other hand, the activity of the purinergic enzyme ADA did not present any significant differences in the serum of patients with AD, compared to unaffected controls [155].

3.6. Parkinson's Disease. Parkinson's disease (PD) is considered the most frequent movement disorder. Clinically, "movement disorder" is defined by its cardinal motor symptoms: bradykinesia, rigidity, and tremor at rest [156]. Furthermore, most patients will also experience nonmotor symptoms that include mood, cognitive, speech, sensation, and sleep disturbances. The major cause of these symptoms is the progressive loss of dopaminergic neurons in the substantia nigra pars compacta projecting to the striatum, leading to a severe deficiency of dopamine in the putamen and the caudate nucleus. The neuropathological hallmark of PD is cytosolic Lewy bodies, which are characterized by aggregated α-synuclein (α-Syn) [157].

Activated microglia is a common feature observed in individuals with neurodegenerative disorders, including PD, and it possibly contributes to neuronal death. Jiang et al. have shown that stimulation of the microglial P2X7 receptor by

extracellular α-Syn, with PI3K/AKT activation and increased oxidative stress, could be an important mechanism and a potential therapeutic target for PD [158]. Furthermore, release of ATP from disrupted cells might cause cell death in neighboring cell-expressing P2X7 receptors, leading to a necrotic volume increase, which has also been implicated in the pathogenesis of PD [159]. Recently, several reports have suggested potential effects of the adenosine A2A receptor antagonist on cognitive dysfunction in PD [160, 161]. Indeed, in rodent models of PD, A2A antagonism exerts antiparkinsonian actions [162–164]. Similarly, this treatment proved to be effective against experimentally induced tremor [165].

Despite several studies provide strong evidence that purinergic receptors are linked to the pathogenesis of PD, the evidence available on a potential involvement of purinergic enzymes is still very scarce. To date, we have only found a few articles concerning this issue. Medeiros et al. have studied the levels of ATP and the activity of the enzymes ADP E-NTPDases, ecto-5′-nucleotidase, and ADA in patients with PD [166]. The results show higher E-NTPDase ATP/ADP activities in PD patients, suggesting an important inflammatory activity in this disease. Another study has analyzed the mRNA levels of E-NTPDase and E-5′-nucleotidase from striatal slices, in a unilaterally lesioned 6-OHDA rat model [167]. The classical method of intracerebral infusion of 6-OHDA involving a massive destruction of nigrostriatal dopaminergic neurons is largely used to investigate motor and biochemical dysfunctions in PD [168]. However, it was found no alteration in the mRNA levels of the enzymes tested [167] (Tables 3 and 4).

3.7. Multiple Sclerosis. Multiple sclerosis (MS) is a chronic inflammatory disease, of progressive character with relapsing-remitting phases, that represents the major disabling neurological illness of young adults [176]. This disease affects the myelin sheath of axons and is characterized by demyelination of neurons in the central and peripheral nervous system. Furthermore, the establishment of tissue oxidative stress associated with a neuroinflammatory process culminates in the loss of myelin content and subsequent death of oligodendrocytes and Schwann cells [177, 178].

The mechanisms implicated in the pathogenesis of MS include autoimmunity, inflammation, demyelination, and neurodegeneration. Since the beginning of demyelination processes, the appearance of multiple plates in the white matter of brain and spinal cord can occur [179]. These lesions lead to the disability or complete loss of nerve impulses that result in the appearance of clinical manifestations such as motor impairment [180], blurred vision, hyperalgesia in the upper and lower limbs, excessive tiredness, weakness, anxiety, depression, and irreversible neurological deficits [179, 181, 182]. The remission is a subsequent phase characterized by the remyelination and tissue repair, with partial or complete disappearance of symptoms [176].

The relationship between inflammation and degeneration during the progression of MS is due to successive outbreaks and remissions. There is a higher frequency of events accompanied inflammatory demyelination of the white matter and damage to axons. In this step, it is possible to tissue repair capacity in which there is a remyelination and axonal in the disappearance of clinical manifestations. As these events are repeated, the tissue recovery becomes ineffective. The secondary progressive phase initiates with the establishment of neurodegenerative tissue followed by cerebral gray matter atrophy. At this stage, there is a progressive increase over the score disabilities that can lead to incapacitation of the patient [183].

The MS does not have etiology and cure defined. Therefore, several types of animal models that produce demyelination have been used for scientific research, in order to discover pharmacological tools for assessing compounds as potential drugs or search for evidence of cellular mechanisms to better understand this disease. Among these, it is possible to highlight the chemically induced CNS lesions, such as those generated with cuprizone feeding, ethidium bromide injection, and lysolecithin, or the experimental autoimmune encephalomyelitis (EAE) model, widely used in drug screening as it most faithfully represents the pathology seen in MS [184]. As described by Merrill, the advantages of these models are the dissociation of the demyelination event, from the complexities introduced into the tissue pathology by chronic inflammatory cells and their soluble mediators, reproducibly timed spontaneous remyelination, and robust demyelination and remyelination in anatomically distinct areas facilitating focused, quantitative assessment of lesion generation and repair [185–187].

Recent reports have highlighted the association between receptors and signaling molecules of the purinergic system with demyelinating diseases, including MS [188–191]. Furthermore, ATP and adenosine also play a significant role in the pathophysiology of numerous acute and chronic disorders that include events related with CNS demyelination and remyelination [23, 192–194].

Over the last decade, the ectonucleotidases have become target of study by modulating purinergic signaling and contributing to the fine-tuning of inflammatory and immune responses. The overwhelming evidence indicates that extracellular ATP acting through specific cell surface receptors is involved in proinflammatory functions such as stimulation and proliferation of lymphocytes and microglial cell and cytokine production and secretion [195, 196]. However, its breakdown product, adenosine, exhibits potent anti-inflammatory and immunosuppressive action by inhibiting proliferation of T cells and secretion of cytokines [197, 198].

The association of the purinergic receptors in the demyelinating diseases has been explored in some previously published studies [199, 200]. An overview of the main findings relating the purinoreceptors with MS in experimental models and postmortem tissue of humans with this pathology can be found in Table 5.

Based on the evidence reported in Table 2, the four purinergic receptors especially known to be involved in MS are P2X7, P2Y12, A1, and A2A receptors. Although the role of purinoreceptors is not well elucidated, it is important to note that the number of published evidence and studies for MS is superior to investigations into the role of

Table 5: Role of purinoreceptors in experimental models and patients with MS: overview of main findings.

Sample	Receptors	Main findings	Reference
Cerebral cortex from healthy and MS patients	P2Y12 receptor	Reduction in the P2Y12R is immunoreactive in the lesions. This event was directly correlated with the extent demyelination found in grey matter cortical and subcortical white matter.	[201]
Tissues of rats exposed to EAE model and brain tissue from healthy and MS patients	P2X7 receptor P2Y12 receptor	P2X7R is highly expressed in microglia in MS lesions during the peak of EAE. P2X7R is associated with a proinflammatory phenotype of human microglia. In parallel, P2Y12R was associated with an anti-inflammatory phenotype in human microglia. P2Y12R was expressed at lower levels in active inflammatory MS lesions. P2Y12R expression increased in the remission phase of EAE.	[202]
Spleen and lymph node cell from P2X7R$^{-/-}$ mice exposed to EAE model	P2X7 receptor	Coculture of P2X7R$^{-/-}$ macrophages with wild-type lymphocytes showed that enhanced proliferative activity resided within the P2X7R$^{-/-}$ lymphocyte population. Furthermore, mRNA and protein for IFN-γ were significantly reduced in the CNS of P2X7R$^{-/-}$ mice with EAE. Enhanced susceptibility of P2X7R$^{-/-}$ mice to EAE reflects a loss of apoptotic activity in lymphocytes.	[203]
EAE induced in rats by guinea pig spinal cord homogenates (GPSCH model)	A1 receptor	Investigation of the role of the A1 receptor using antagonists. Caffeine (10–30 m/kg) decreases the incidence of EAE and attenuates EAE pathology at behavioral, histological (inflammatory cell infiltration and demyelination), and neurochemical (expression of inflammatory cytokines) levels. In addition, caffeine also upregulated A1 receptor and TGF-β mRNAs and suppressed INF-γ mRNA in EAE rats.	[204]
Brain of female Lewis rats exposed to EAE model	P2X7 receptor	Enhanced expression of GFAP and S100β is associated with expression of P2X7R. Brilliant blue G, an antagonist of P2X7R, significantly decreases astrogliosis (GFAP and S100β).	[205]
Brain of rats exposed to EAE model	P2X7 receptor	The enhancement in the expression of the P2X7 receptor at the level of both mRNA and protein was observed in the peak of neurological symptoms and was connected mostly with neurons (4, 6, 8, and 10 days postimmunization).	[206]
Human monocytes from Australasian patients with MS	P2X7 receptor	A rare P2X7 variant Arg307Gln with absent pore formation function protects against neuroinflammation in MS.	[207]
Two independent mouse EAE models: by immunization in C57BL/6 using MOG_{35-55} and by $PLP_{139-155}$ in mouse wild-type and lacking A2AR (A2AR$^{-/-}$)	A2A receptor	Upregulation of A2AR in the CNS in EAE, predominantly detected on T cells and macrophages/microglia. A preventive EAE treatment with A2AR-specific agonist inhibited myelin-specific T cell proliferation ex vivo and ameliorated disease. In parallel, the application of the same agonist after disease onset exacerbated nonremitting EAE progression and resulted in more severe tissue destruction. A2AR-deficient mice showed accelerated and exacerbated disease manifestation with higher numbers of inflammatory lesions in the early stage. EAE quickly ameliorated and myelin debris accumulation was lower in A2AR$^{-/-}$ mice. Finally, an in vitro activation of A2AR inhibited phagocytosis of myelin by macrophages and primary microglia as well as migration of CD4$^+$ T cells, macrophages, and primary microglia.	[194]
Lymphocyte isolation from nerve tissue and lumbar spinal cord of female mice exposed to EAE	A2A receptor	CGS21680 (CGS, A2AR agonist) significantly suppressed specific lymphocyte proliferation, reduced infiltration of CD4$^+$ T lymphocytes, and attenuated the expression of inflammatory cytokines, which in turn inhibited the EAE progression. CGS can increase the [Ca2$^+$]i in murine lymphocytes, which may be the mechanism underlying the suppressive effects of CGS-induced A2AR activation on EAE progression.	[208]
Oligodendrocyte cultures and postmortem optic nerve samples from MS patients	P2X7 receptor	Sustained activation of P2X7R in vivo causes lesions that are reminiscent of the major features of MS plaques (demyelination, oligodendrocyte death, and axonal damage). In addition, treatment with P2X7R antagonists reduces demyelination and ameliorates the associated neurological symptoms. The study	[209]

Table 5: Continued.

Sample	Receptors	Main findings	Reference
		suggests that ATP can kill oligodendrocytes via P2X7R activation and this process contributes to EAE. Importantly, P2X7R expression is elevated in normal-appearing axon tracts in MS patients.	
Peripheral blood mononuclear cells from MS patients	A1 receptor	Decreased levels of adenosine and its A1 receptor modulate TNFα and IL-6 levels and may contribute to the pathogenesis of MS.	[210]
Brain and spinal cord of female SJL/J mice infected with Theiler's virus infection	A2A receptor	A2A receptors participate in anti-inflammatory effects of cannabidiol. A2A antagonist ZM241385 partially blocks the protective effects of cannabidiol in the initial stages of inflammation.	[211]
Human microglia	P2Y12 receptor	P2Y12 is expressed on parenchymal microglia and is stable throughout human brain development, including fetal phases. MS result in decreased P2Y12 immunoreactivity in plaque- or lesion-associated myeloid cells. P2Y12 is a useful marker for the identification of human microglia throughout the lifespan.	[212]
Blood from MS patients	P2X4 receptor P2X7 receptor	A rare genetic variant in P2RX4 and P2RX7 is a major genetic contributor to disease (description of the three variant haplotypes: P2RX7 rs140915863:C>T [p.T205M]; P2RX7 rs201921967:A>G [p.N361S]; and P2RX4 rs765866317:G>A [p.G135S]).	[213]
C57BL6 mice and P2X7-deficient mice exposed to EAE model	P2X7 receptor	The incidence of EAE disease in P2X7 mice was reduced 4-fold compared to the wild type. Mouse splenic T cells isolated from P2X7 null mice produced greater IFNγ and IL-17 (from 3- to 12-fold greater levels) than wild-type cells. Although infiltrating cells were detected in the brains of both the P2X7 and wild type, astroglial activation and axonal damage were reduced compared to wild type.	[214]
Brain and spinal cord from female mice exposed to EAE model	A1A receptor	$A1AR^{-/-}$ mice developed a severe progressive-relapsing form of EAE compared with their wild type. Demyelination, axonal injury, and enhanced activation of microglia and macrophages were observed in $A1AR^{-}/^{-}$. Spinal cords from $A1AR^{-/-}$ mice demonstrated increased proinflammatory gene expression. $A1AR^{-/-}$ macrophage-derived soluble factors caused significant oligodendrocyte cytotoxicity compared with wild-type controls.	[215]
Spinal cord from MS patients	P2X7 receptor	In control spinal cord, few small microglial cells/macrophages were scattered throughout the tissue. However, MS specimens had significantly greater density of such cells with longer processes in affected regions. MS also had significantly greater density of P2X7 and immunoreactive microglial cells/macrophages in affected regions.	[216]

MS (multiple sclerosis), experimental autoimmune encephalomyelitis (EAE), central nervous system (CNS), and glial fibrillary acid protein (GFAP).

ectonucleotidases in this pathology. In this way, in Table 2, we also reviewed the activity and expression of ectonucleotidase enzymes ecto-NTPDase, E-5′-nucleotidase, and adenosine deaminase in experimental models and in patients with MS (see Table 6).

4. Conclusions

The main causes of death and sickness around the world have changed significantly in the last years, with chronic neurologic and degenerative diseases becoming more and more important. Several evidences show that purinergic signaling system is involved in processes associated with health and disease. In this way, the role of the purinergic receptors in the pathophysiology of different degenerative diseases has been extensively studied. However, the role of the purinergic system enzymes is not fully understood yet. This review summarizes the most current knowledge on the role of families of nucleotide metabolizing enzymes, the ectonucleotidases, on degenerative diseases. Considerable attention was directed towards diseases related to the cardiovascular system (atherosclerosis and hypertension) and central nervous system (epilepsy, Alzheimer's disease, Parkinson's disease, and multiple sclerosis). Firstly, it has been shown that a reduction in the activity of ectonucleotidases can be associated with progression of arteriosclerosis. Furthermore, high expression and activity of CD73 and E-NTPDase 1 may have beneficial effects contributing to the production of extracellular adenosine. In parallel, the low expression and activity of ADA are an alternative maintenance of levels of adenosine,

Table 6: Ectonucleotidases (E-NTPDase, E-5′-nucleotidase, and ADA) in experimental models and patients with MS: a review.

Sample	E-NTPDase	E-5′-nucleotidase	Adenosine deaminase	Reference
Serum of MS patients (RRMS form)	—	—	↑ enzymatic activity	[169]
Lymphocytes of MS patients (RRMS form)	↑ expression CD39 ↑ enzymatic activity to ATP and ADP	—	↓ enzymatic activity	[170]
Platelets of MS patients (RRMS form)	↓ enzymatic activity to ATP and ADP	↓ enzymatic activity	↓ enzymatic activity	[171]
Platelets from rats demyelinated with EB	↓ enzymatic activity to ATP (on days 3, 7, 15, and 21) ↓ enzymatic activity to ADP (on day 7)	↓ enzymatic activity (on day 15)	—	[172, 173]
Platelets from rats demyelinated with EB	↑ enzymatic activity to ATP (on day 7) ↑ enzymatic activity to ADP (on day 7)	↑ enzymatic activity (on day7)	—	[174]
Synaptosomes from cerebral cortex of rats demyelinated with EB	↑ enzymatic activity to ATP (on days 7 and 15) ↑ enzymatic activity to ADP (on day 7)	↑ enzymatic activity (on day 7)	—	[174, 175]
Plasma membrane from lumbosacral region of spinal cords from EAE-induced rats	↑ enzymatic activity to ATP (on days 7, 15, and 25) ↑ enzymatic activity to ADP (on day 15)	↑ enzymatic activity (on days 15 and 25)	—	[217]
Blood serum from EAE-induced rats	↑ enzymatic activity to ATP (on day 25) ↓ enzymatic activity to ADP (on days 15 and 25)	↓ enzymatic activity (on day 15)	—	[217]
T cells from patients with relapsing-remitting MS	↓ in number of CD39-positive Treg cells	—	—	[218, 219]

EB (ethidium bromide), relapsing-remitting multiple sclerosis (RRMS), experimental autoimmune encephalomyelitis (EAE), and Treg (regulatory T cells).

in order to increase antithrombotic and anti-inflammatory effects. In relation to hypertension, a suppression of E-NTPDase 1 is linked to the pathogenesis of this disease and an increase in the activity of ectonucleotidases was reported in animal models of hypertension and in human. On the other hand, P2 receptor modulation seems to be a promising tool for a novel therapeutic approach for epilepsy. Lastly, a reduction of ATP levels in experimental models for AD and increase in the ectonucleotidases activities, especially E-NTPDase in patients with Parkinson's disease and multiple sclerosis, were found.

In conclusion, we verified that the activity and expression of the ectonucleotidases investigated in this review may be altered in some degenerative diseases. Taken together, the data discussed in this paper collaborate for a better understanding of the molecular mechanisms involved in these highly complex diseases, suggesting a number of directions for future research. In fact, more studies should be conducted to better understand the changes in metabolism and maintenance of adenine nucleotide and nucleoside levels in degenerative diseases. In addition, the study of the ectonucleotidase role may contribute to a better understanding of the molecular mechanisms of highly complex diseases and with major impact on patients' quality of life.

5. Future Prospects

Ectonucleotidases are nucleotide-metabolizing enzymes and play an essential role in regulating and controlling extracellular nucleotides. These nucleotides and nucleosides are responsible for the well functioning of normal cells. A wrong signaling mediated by these molecules triggers pathophysiological disorders like as atherosclerosis, hypertension, cancer, epilepsy, Alzheimer's disease, Parkinson's disease and multiple sclerosis. In this way, understanding the role of these molecules can be an important key factor to discover pharmacological targets and mechanisms that minimize deleterious effects of these diseases.

Until now, the investigations describes which ectonucleotidases have their activity or expression changes in different biological tissues in experimental models and patients with the pathologies reviewed in this study. From these findings, the next step is to know how the catabolic activity of ectonucleotidases can regulate the production of proinflammatory mediators and the immune response, both physiologically and pathologically.

Evidences have pointed out the important role of inhibitors of ectonucleotidases, highlighting their potential as novel drugs [220–222]. It is suggested that inhibitors of

ectonucleotidases might be an ideal drug target for many therapeutic applications such as anticancer and immunomodulatory for the treatment of central nervous system disorders and cardiovascular diseases [220]. An important perspective would be to explore the use of these drugs in experimental biology. Such investigations would reveal whether up- or downregulation of the ectonucleotidases would restore immune markers and physiological functions compromised in experimental models and cell cultures. Since inhibitors of ectonucleotidases might be an ideal drug target for many therapeutic applications such as anticancer and immunomodulatory for the treatment of central nervous system disorders and cardiovascular diseases [220], more research is needed to elucidate the role of these enzymes in these pathologies and their role potential targets for drug discovery.

Conflicts of Interest

All authors report no conflicts of interest.

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Research Article

Influence of Biological Therapeutics, Cytokines, and Disease Activity on Depression in Rheumatoid Arthritis

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Purpose. Rheumatoid arthritis (RA) is an often debilitating autoinflammatory disease. Patients with rheumatoid arthritis are often troubled by co-occurring depression or other psychological manifestations. RA patients have a variety of treatment options available, including biologicals that inhibit cytokines or immune cells. If these cytokines influence the psychological symptoms, then the use of cytokine inhibitors should modulate these symptoms. *Methods.* A cohort of 209 individuals was recruited. This group included 82 RA patients, 22 healthy subjects, 32 depressed control subjects, and 73 subjects with systemic lupus erythematosus. Of the RA patients, 51% were on a biological therapeutic. ELISA was used to measure cytokine levels. A variety of psychological assessments were used to evaluate depression, anxiety, sleep, fatigue, and relationship status. Clinical values were obtained from medical records. *Results.* IL-10 concentration was associated with depressive symptoms in the RA patients, healthy controls, and the lupus patients. In the patients with primary depression, depressive symptoms were associated with IL-6 and TNF-alpha. In RA patients, Tocilizumab use was associated with decreased depressive symptoms. 14 RA patients who were not using biologicals began using them by a one-month follow-up. In these patients, there was no significant change to any value except for fatigue. *Conclusions.* A variety of both biological and social factors influences depressive symptoms in RA. IL-10 and IL-6 are likely to be involved, since IL-10 concentration was associated with depression and Tocilizumab decreased depressive symptoms in the RA patients. The roles of these cytokines are different in RA and lupus, as high IL-10 in RA is associated with increased depressive symptoms, but high IL-10 in the lupus patients is associated with decreased depression. IL-6 was also associated with depressive symptoms in the patients with primary depression. These results strongly indicate that disease activity, including cytokine levels, has a strong impact on depressive symptoms.

1. Background

Rheumatoid arthritis (RA) is a destructive autoinflammatory arthritis characterized by the overproduction of certain pro-inflammatory cytokines. The disease is destructive in nature, leading to pain, stiffness, bone resorption, and systemic inflammation. Treatment with monoclonal antibodies or antibody derivatives (biologicals) that block specific cytokines has been very successful. Many of these biologicals prevent bone destruction and have a substantial impact on

disease activity [1]. Biologicals are also useful in understanding the mechanisms of rheumatoid arthritis, since they block the activity of specific cytokines.

Patients with rheumatoid arthritis have a higher prevalence of depression and anxiety than the general population [2]. Part of this increased prevalence may be due to the toll that chronic, painful disease takes on the quality of life. Another aspect may be the heightened levels of inflammatory cytokines found in rheumatoid arthritis. Several of these cytokines have been linked to depression, such as TNF-alpha [3–6] and IL-6 [7]. IL-10 is often negatively correlated with depression [8, 9]. Depression may influence cytokine activity, as patients with more depressive symptoms are less responsive to biological antirheumatic therapies [10].

In order to investigate the role of cytokines in depression associated with rheumatoid arthritis, we studied the relationship between depression, clinical markers, and serum cytokine levels in rheumatoid arthritis patients, patients being treated for primary depression, healthy controls, and lupus patients. We further hypothesized that cytokine inhibition via biological agents would have an impact on depressive symptoms. To test this hypothesis, a subcohort of rheumatoid arthritis patients being treated with biological therapeutics to inhibit specific cytokines was examined, to determine the effect of cytokine inhibition on depression.

2. Materials and Methods

A cohort of 209 individuals was recruited for this study. This group included 82 RA patients, 22 healthy control subjects, 32 subjects with primary depression, and 73 subjects with systemic lupus erythematosus as a control group for autoimmune arthritis (Table 1). Diagnosis and stage of disease activity were established according to the American College of Rheumatology Classification Criteria (ACR) [11], by a rheumatologist. All RA patients attended routine visits at the hospital and completed regular clinic and laboratory assessments. Patients with depressive disorder were diagnosed by a psychiatrist working in a psychiatric private clinic according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR). Female healthy controls were recruited using a snowball approach and included in the sample. Patient recruitment was made during a consultation. Once accepted, the researcher further informed the participants in a separate room of the study aims and procedures and all who agreed to participate received written and verbal information about the study and signed an informed consent form and were invited to perform a peripheral blood collection. A telephone call was scheduled to perform the psychosocial evaluation, and recruited patients and controls were subsequently interviewed by phone by a psychologist. The literature corroborates phone interviews as valid and precise tools for psychological data collection. [12]. Laboratory and clinical evaluations were obtained for the RA and lupus patients through clinical records. Lab tests included leukocytes (10^9/L), lymphocytes (percentage), platelets (10^9/L), erythrocyte sedimentation rate (mm/h), anti-dsDNA antibody titer (IU/mL), and C-reactive protein level (mg/dL). Disease activity was assessed by both the patient and the doctor, and the DAS28 was used for overall evaluation. Physical activity, smoking, and alcohol consumption were also recorded. Physical activity was assessed based on involvement in sporting activities.

The study was submitted and approved by the Ethical Committee of the São João Hospital IRB (EPE) in accordance with the Declaration of Helsinki. The nature and the purpose of the study were explained to all participants who signed the informed consent form before they entered the study.

2.1. Psychosocial and Clinical Evaluation. Sociodemographic characteristics were included in the questionnaire. Education level was given as years of school. Age and marital status were listed. Socioeconomic class evaluation was included using employment status and years of education as proxies. Psychological evaluations were performed using several standardized instruments.

2.1.1. Fatigue Severity Scale (FSS). The short form of the FSS was used for this study. This form allows the participant to report their perceived level of fatigue [13]. The Portuguese version includes nine items. This instrument has been found to be valid and useful for studies involving patients with systemic lupus erythematosus [14].

The Cronbach α is 0.89 for the FSS, and the test-retest reliability is 0.84, which indicates good psychometric properties. The mean of all scored items is used to generate a total score, with higher scores indicating a higher severity of fatigue. Clinical fatigue is defined by a FSS score > 3. The scale has is sensitive to change, and interviews conducted via telephone can use it reliably. The FSS has also been shown to be reliable across different patient populations [15].

2.1.2. Hospital Anxiety and Depression Scale (HADS). The Hospital Anxiety and Depression Scale (HADS) measures anxiety and depression, especially in people with physical illness [16]. The scale has a Cronbach alpha coefficient of 0.94, indicating good psychometric properties [17]. The HADS is subdivided into two subscales of 7 items. One subscale measures depression and one measures anxiety. The scores for of each scale can be between 0 and 21. Scores ranging from 8 to 10 are considered mild, from 11 to 14 moderate, and 15 to 21 severe [18]. For this study, 8 is used as the point indicating either anxiety or depression [16]. It is important to note that the scale shows symptoms of depression or anxiety in the last week and therefore does not necessarily indicate clinical depression.

2.1.3. Pittsburgh Sleep Quality Index (PSQI). The PSQI is a measurement of self-assessed sleep quality. High PSQI scores show low sleep quality, and low PSQI scores mean high sleep quality. Reliability of the PSQI is high, with a Cronbach alpha of 0.83. Seven components of sleep are evaluated by this instrument. These include sleep latency, sleep disturbances, sleep duration, sleep quality, sleep efficiency, use of sleep medications, and daytime dysfunction. The scores for each of these components are combined for a global score that can range from 0 to 21 [19]. An overall score from zero to five is normal sleep, while an overall score of greater than five is indicative of poor sleep quality [20].

Table 1: Sociodemographics of cohort.

Characteristics	All subjects ($N = 209$)	RA subjects ($N = 82$)	Healthy subjects ($N = 22$)	p value	Depressed subjects ($N = 32$)	p value	SLE subjects ($N = 73$)	p value
Gender, number (%)								
Female	192 (92)	69 (84)	21 (95)	0.291	29 (91)	0.550	73 (100)	<0.001
Age, mean ± SD	49.27 ± 11.99	54.99 ± 10.08	43.14 ± 11.54	< 0.001	49.25 ± 14.03	0.040	44.70 ± 10.38	<0.001
Education (years), mean ± SD	8.43 ± 4.45	6.85 ± 3.99	13.55 ± 3.10	< 0.001	8.91 ± 4.31	0.017	8.88 ± 4.14	0.002
Education level, number (%)				< 0.001		0.200		<0.001
Primary (or less)	97 (46)	53 (65)	1 (5)		15 (47)		28 (38)	
Middle school	34 (16)	14 (17)	2 (9)		7 (22)		11 (15)	
High school	44 (21)	7 (9)	8 (36)		5 (16)		24 (33)	
College	32 (15)	7 (9)	10 (45)		5 (16)		10 (14)	
Graduate school	2 (1)	1 (1)	1 (5)		0 (0)		0 (0)	
Marriage status, number (%)				0.024		0.428		0.026
Unmarried	33 (16)	9 (11)	8 (36)		6 (19)		10 (14)	
Married	135 (65)	50 (61)	9 (41)		22 (69)		54 (74)	
Divorced	28 (13)	14 (17)	4 (18)		2 (6)		8 (11)	
Widowed	10 (5)	8 (10)	0 (0)		2 (6)		0 (0)	
Cohabiting	3 (1)	1 (1)	1 (5)		0 (0)		1 (1)	
Employment status, number (%)				0.002		0.137		0.452
Employed	88 (42)	28 (34)	16 (73)		16 (50)		28 (38)	

2.1.4. Relationship Assessment Scale (RAS). The RAS [21] (Portuguese experimental version from Mesquita, Barbosa, and Figueiredo-Braga, 2014) is a 7-item instrument, with a five-point scale that measures general satisfaction with the marital or intimate relationship.

2.1.5. Disease Activity Score 28 (DAS28). The Portuguese version of the DAS 28 [22] was used to measure disease activity in RA patients. This index is calculated through joint examination, blood tests of inflammation, and global pain assessment. The lower the DAS28 score, the better the controlled disease [23].

2.1.6. Pain Scale. Pain was self-reported to the telephone interviewer using a numerical scale from 1 to 10.

2.2. Cytokine Analysis. ELISA kits were purchased from eBiosciences, and serum cytokine levels were measured according to the manufacturer's directions. Peripheral blood samples were always collected in the morning (9–11 a.m.) into plastic tubes containing K3EDTA as an anticoagulant for plasma determinations or a gel separator for serum assays (BD Vacutainer, Franklin Lakes, NJ). Blood was centrifuged at 2000g for 10 minutes. Serum and plasma were frozen at −70°C for further determinations. Levels of IL-6, IL-10, and TNF-alpha were measured using ELISA for each participant.

2.3. Statistical Analysis. Significant differences in the demographical, clinical, and psychological variables between the SLE subjects, healthy controls, RA subjects, and depressed control subjects were determined using the independent t-test, Fisher's exact chi-squared, Mann–Whitney U, Wilcoxon rank sum, or Welch's tests when considered appropriate. Fisher's exact chi-squared was used in place of the standard chi-squared test, which would typically be utilized, due to the smaller sample size of the groups compared. The statistical tests used for the comparisons are indicated in the table legends.

Univariate analysis was accomplished by using generalized linear or logistic regression, using the Poisson function when appropriate, due to the Poisson distribution observed in the data. HADS depression scores were utilized as the dependent variable and with the individual variable suspected of showing a correlation to depression as the independent variable. Statistical analysis was performed using the statistical software R and SPSS (IBM). The multivariate analysis of the RA cohort was accomplished using logistic regression utilizing the Poisson function. HADS depression was used as the dependent variable. Only independent variables that were not co-related and had demonstrated a significant association in univariate analysis were used in the multivariate models to generate the best fit model. The best fit model was chosen based on the model demonstrating the lowest AICc value with the highest pseudo R^2 value.

To compare the rheumatoid arthritis patients that began biological therapy during the second month of the study, the appropriate paired t-test was utilized based on the data. For categorical variables, Fisher's exact test was used for the reasons previously described. An alpha value less than or equal to 0.05 was considered significant in all analyses.

2.4. Power Analysis. A post hoc power analysis was performed to test for the likelihood of type 2 errors, in which

true correlations are missed due to inadequate sample size. The advantage of a post hoc power analysis is that it can consider the actual variation seen in the sample, as opposed to a projected variance, and thus provide a realistic analysis (Supplementary Table 1).

3. Results

Four different groups were recruited for this study: rheumatoid arthritis patients, lupus patients, primary depression patients, and healthy controls. The rheumatoid arthritis patients were significantly older than the other groups. They also had fewer years of education than the other groups. The RA patients were more likely to be married than the healthy controls, but less likely than the lupus patients. They were also less likely to be employed than the healthy controls, but not than the primary depression or lupus patients (Table 1). However, multivariate analysis revealed that these factors were not among the most significant contributors to depressive symptoms in rheumatoid arthritis. Univariate analysis was used to identify correlations between depression and clinical, treatment, and social factors (Tables 2–6). Multivariate analysis was used to identify the variables that most strongly predicted depression in the RA patients (Table 7).

Depressive symptoms were assessed for all participants using the HADS instrument. Therapeutics, clinical indicators, and psychological factors were all examined for correlation with symptoms of depression. Multiple correlations were found, encompassing all three areas. The use of the four different participant groups (rheumatoid arthritis, lupus (SLE), depressive disorder, and healthy controls) allowed the evaluation of the relative importance of each factor in rheumatoid arthritis. For example, if a particular condition correlated with depression in RA but not SLE, that would indicate that the condition was specific for RA and not a general feature of autoimmune arthritis.

3.1. Biological Therapeutics and Depression. In RA patients, the use of Tocilizumab, an IL-6 inhibitor, was associated with decreased depressive symptoms ($P = 0.023$, with an odds ratio of 0.70). No other biological agent was associated with depressive symptoms in either a positive or a negative manner. Interestingly, Sulfasalazine, a nonbiological treatment, was also associated with decreased depression ($p = 0.012$. OR = 0.58) (Table 2).

Fourteen RA patients were not initially on a biological therapeutic but were being treated with one at the time of the one-month follow-up. Of these patients, there was no significant change in depressive symptoms, although counter to what might be expected, they reported significantly higher fatigue ($p = 0.01$) in the follow-up month than in the first assessment. This fatigue was present even though there was no significant change in disease activity.

3.2. Cytokines and Depression. Levels of IL-6, IL-10, and TNF-alpha were measured using ELISA for each participant. IL-10 concentration correlated with depressive symptoms in patients with rheumatoid arthritis ($p = 0.005$, OR = 1.13), in that higher IL-10 levels correlated with more depressive symptoms. This correlation was also found in the healthy controls. Even though these individuals were not diagnosed with autoimmune disease or depression, IL-10 concentrations still correlated with depressive symptoms, although the association was not terribly strong ($p = 0.047$, OR = 1.05). However, in the patients with lupus, IL-10 levels were inversely correlated with depressive symptoms ($p = 0.006$, OR = 0.92), as has been seen for other diseases.

IL-6 and TNF-alpha correlations were also identified, although only in the primary depression patients. In these patients, IL-6 and TNF-alpha were both positively correlated with depressive symptoms ($p = 0.011$ and OR = 1.07 and $p = 0.048$ and OR = 1.00, resp.) (Table 3).

3.3. Clinical Values and Depression. Disease activity was strongly correlated with depression in the RA patients in this study. Both patient and physician assessments of overall disease activity strongly correlated with depressive symptoms ($p < 0.001$ for both measures) (Table 4). Clinical test results also correlated with depression in the RA patients. Sedimentation rate, C-reactive protein levels, and DAS28 scores all positively correlated with depressive symptoms (Table 4). In the lupus patients, these measures of inflammation did not correlate with depressive symptoms (Table 5).

Certain physical factors also correlated with depression. In both the RA patients and the lupus patients, body mass index correlated strongly with depressive symptoms ($p < 0.001$ and $p = 0.003$, resp.). BMI did not correlate with depressive symptoms in the patients with primary depression or in the healthy controls. Physical activity was strongly negatively correlated with depressive symptoms in rheumatoid arthritis ($p < 0.001$) and lupus ($p = 0.047$) (Tables 4 and 5). In fact, low physical activity is one of the factors identified by the multivariate analysis as being the most strongly predictive of depression using multivariate analysis (Table 7).

3.4. Pain, Psychosocial Measurements, and Depression. Self-reported pain scores correlated with depression in patients with RA ($p < 0.001$), lupus ($p < 0.001$), and primary depression ($p < 0.001$). Fatigue, relationship quality, and sleep quality were assessed for each study participant. All three of these measurements were strongly correlated with symptoms of depression in the RA patients ($p < 0.001$ for all). Therefore, depression in RA was correlated with worse overall sleep quality, poor relationship quality, and increased fatigue. Variants on this pattern were also observed for the other groups. Depression in lupus was correlated with fatigue ($p < 0.001$) and overall sleep quality ($p = 0.012$), but not marital relationship quality. In the depressive disorder patients, symptoms of depression were also associated with overall sleep quality ($p = 0.008$) and fatigue ($p < 0.001$). In the healthy controls, marital relationship quality was inversely associated with active depression ($p = 0.009$), and fatigue was directly associated ($p = 0.005$) (Table 6).

3.5. Multivariate Analysis. Multivariate analysis was performed to identify which of the variables was the most predictive for depressive symptoms and to ensure that

Table 2: Depressive symptoms correlated with medication in rheumatoid arthritis.

Medication	Pseudo R^2	AICc	Coefficient	Odds ratio (95% CI)	p value
Biological medications	0.154	572.98			
None			0.059	1.06 (0.83–1.37)	0.643
Abatacept			−0.143	0.87 (0.45–1.52)	0.642
Adalimumab			−0.143	0.87 (0.45–1.52)	0.642
Etanercept			0.011	1.01 (0.72–1.41)	0.948
Golimumab			0.011	1.01 (0.43–2.03)	0.978
Infliximab			0.113	1.12 (0.73–1.66)	0.588
Rituximab			0.145	1.16 (0.85–1.57)	0.361
Tocilizumab			−0.351	0.70 (0.52–0.95)	0.023
Classic medications	0.829	478.81			
Leflunomide			−0.284	0.75 (0.56–1.01)	0.061
Methotrexate			−0.207	0.81 (0.62–1.06)	0.125
Leflunomide and Methotrexate			0.301	1.35 (0.82–2.12)	0.209
Sulfasalazine			−0.546	0.58 (0.37–0.87)	0.012
Hydroxychloroquine + Leflunomide + Methotrexate			−0.441	0.64 (0.31–1.17)	0.184
Hydroxychloroquine + Methotrexate			0.090	1.09 (0.73–1.60)	0.650
Cyclosporine			0.029	1.03 (0.46–1.98)	0.937
Hydroxychloroquine + Leflunomide			−0.258	0.77 (0.30–1.61)	0.539
Sulfasalazine + Methotrexate			0.147	1.16 (0.70–1.86)	0.565
Hydroxychloroquine			0.147	1.16 (0.68–1.86)	0.565
Sulfasalazine + Hydroxychloroquine			0.147	1.16 (0.68–1.86)	0.565
Sulfasalazine + Leflunomide			−1.357	0.26 (0.04–0.81)	0.057

Table 3: Correlations between depressive symptoms and serum cytokine levels.

		Pseudo R^2	AICc	Coefficient	Odds ratio (95% CI)	p value
Rheumatoid arthritis	($n = 82$)					
IL-6 (pg/mL), mean ± SD	44.04 ± 104.39	1.000	73.80	0.002	1.00 (1.00-1.00)	0.132
IL-10 (pg/mL), mean ± SD	4.12 ± 2.97	1.000	68.99	0.121	1.13 (1.03–1.22)	0.005
TNF-alpha (pg/mL), mean ± SD	109.92 ± 151.85	1.000	75.19	0.001	1.00 (1.00-1.00)	0.430
Lupus	($n = 73$)					
IL-6 (pg/mL), mean ± SD	2.81 ± 2.18	1.000	83.69	0.055	1.06 (0.96–1.15)	0.229
IL-10 (pg/mL), mean ± SD	6.76 ± 4.06	1.000	76.47	−0.088	0.92 (0.86–0.97)	0.006
TNF-alpha (pg/mL), mean ± SD	60.66 ± 102.38	1.000	83.56	−0.001	1.00 (1.00-1.00)	0.243
Depressive disorder	($n = 32$)					
IL-6 (pg/mL), mean ± SD	1.30 ± 2.23	0.970	143.99	0.070	1.07 (1.01–1.13)	0.011
IL-10 (pg/mL), mean ± SD	9.18 ± 15.66	0.962	146.13	0.008	1.01 (1.00-1.02)	0.059
TNF-alpha (pg/mL), mean ± SD	38.43 ± 67.75	0.962	146.01	0.002	1.00 (1.00-1.00)	0.048
Healthy controls	($n = 22$)					
IL-6 (pg/mL), mean ± SD	1.11 ± 1.34	0.653	102.02	0.014	1.01 (0.84–1.18)	0.869
IL-10 (pg/mL), mean ± SD	3.07 ± 3.78	0.712	98.70	0.047	1.05 (1.00-1.09)	0.047
TNF-alpha (pg/mL), mean ± SD	33.77 ± 53.08	0.706	99.05	−0.005	1.00 (0.99–1.00)	0.109

differing sociodemographic factors between groups were not responsible for the outcomes of the study. This analysis showed that none of the sex, age, marital status, or years of education significantly predicted depressive symptoms. The best correlations with depressive symptoms were found for low physical activity ($p = 0.003$), poor relationship satisfaction as measured by the RAS ($p < 0.001$), and fatigue as measured by the FSS ($p < 0.001$) (Table 7).

Table 4: Correlations between depressive symptoms and clinical assessments in RA subjects.

	($n = 82$)	Pseudo R^2	AICc	Coefficient	Odds ratio (95% CI)	p value
Characteristics						
Disease duration (years), mean ± SD	17.56 ± 11.48	0.252	461.64	−0.040	0.96 (0.88–1.05)	0.401
BMI, mean ± SD	26.46 ± 5.49	0.144	557.56	0.028	1.03 (1.01–1.04)	< 0.001
Pain score, median	6	0.411	527.31	0.114	1.12 (1.08–1.16)	< 0.001
Smoking, number (%)	0 (0)	< 0.001	570.11	−0.029	0.97 (0.77–1.22)	0.806
Drinking, number (%)	13 (16)	0.014	569.01	−0.129	0.88 (0.69–1.11)	0.290
Physical activity, number (%)	13 (16)	0.256	546.22	−0.668	0.51 (0.38–0.68)	< 0.001
Biologic medication, number (%)	42 (51)	0.027	570.09	−0.082	0.92 (0.73–1.18)	0.503
Classic medication, number (%)	55 (67)	0.030	569.89	−0.062	0.94 (0.75–1.19)	0.601
Disease activity (patient), mean ± SD	54.04 ± 30.16	0.880	426.21	0.002	1.01 (1.01–1.02)	< 0.001
Disease activity (doctor), mean ± SD	35.44 ± 27.03	0.890	419.81	0.013	1.01 (1.01–1.02)	< 0.001
Laboratory						
Sedementation velocity, mean ± SD	26.59 ± 22.27	0.834	448.13	0.010	1.01 (1.01–1.01)	< 0.001
C-reactive protein, mean ± SD	11.11 ± 17.43	0.778	468.09	0.006	1.01 (1.00-1.01)	0.007
DAS28, mean ± SD	5.04 ± 5.65	0.830	449.81	0.038	1.04 (1.02–1.05)	< 0.001
Brain leukocytes, mean ± SD	8.09 ± 3.40	0.707	484.34	0.010	1.01 (0.98–1.04)	0.425
Brain lymphocytes, mean ± SD	27.10 ± 9.84	0.713	482.76	−0.007	0.99 (0.98–1.00)	0.139
Anti-dsDNA, mean ± SD	10.66 ± 5.30	0.999	248.28	0.017	1.02 (1.00-1.04)	0.069

Table 5: Correlations between depressive symptoms and SLE clinical assessments.

			SLE subjects			
	($n = 73$)	Pseudo R^2	AICc	Coefficient	Odds ratio (95% CI)	p value
Characteristics						
Disease duration (years), mean ± SD	17.81 ± 8.85	0.021	467.95	0.006	1.01 (1.00-1.02)	0.211
BMI, mean ± SD	24.51 ± 5.60	0.103	461.59	0.021	1.02 (1.01–1.03)	0.003
Smoking, number (%)						
Yes	13 (18)	0.032	467.14	−0.186	0.83 (0.65–1.05)	0.133
Drinking, number (%)						
Yes	7 (10)	0.006	469.03	0.100	1.11 (0.82–1.45)	0.489
Physical activity, number (%)						
Yes	24 (33)	0.054	465.43	−0.196	0.82 (0.68–0.99)	0.047
Laboratory						
Sedimentation velocity, mean ± SD	23.48 ± 20.96	0.355	440.12	0.003	1.00 (1.00-1.01)	0.220
C-reactive protein, mean ± SD	0.44 ± 0.57	0.191	454.67	0.120	1.13 (0.97–1.29)	0.094
Leukocytes, mean ± SD	6.73 ± 2.13	0.160	457.26	−0.003	1.00 (0.96–1.04)	0.884
Brain leukocytes, mean ± SD	6.22 ± 2.20	1.000	91.44	0.121	1.13 (1.04–1.23)	0.005
Lymphocytes, mean ± SD	28.89 ± 8.09	0.160	457.27	0.001	1.00 (0.99–1.01)	0.929
Brain lymphocytes, mean ± SD	29.05 ± 6.77	1.000	96.64	−0.022	0.98 (0.95–1.01)	0.117
Anti-dsDNA, mean ± SD	98.79 ± 111.01	0.829	370.64	< 0.001	1.00 (1.00-1.00)	0.990
Brain anti-dsDNA, mean ± SD	635.54 ± 1504.14	1.000	96.28	< 0.001	1.00 (1.00-1.00)	0.081

4. Discussion

It is evident from these results that depression in rheumatoid arthritis is strongly linked to disease activity, as nearly every measure of systemic inflammation and disease activity was correlated with depressive symptoms. This is not the case in systemic lupus, where disease activity often bears little relation to depressive symptoms. Of the clinical indicators, only C-reactive protein levels were associated with active depression in lupus. This difference suggests that the root cause of depression is different in rheumatoid arthritis and lupus.

The results of the cytokine analysis support this finding. IL-10 is associated with depression in both lupus and

Table 6: Correlations between depression, pain, and psychosocial values.

		Pseudo R^2	AICc	Coefficient	Odds ratio (95% CI)	p value
Rheumatoid arthritis	($n = 82$)					
Pain score, median	6	0.411	527.31	0.114	1.12 (1.08–1.16)	<0.001
PSQI, mean ± SD	8.09 ± 4.29	0.442	523.54	0.065	1.07 (1.05–1.09)	<0.001
Relationship Assessment Scale, mean ± SD	28.74 ± 9.54	0.989	328.73	−0.041	0.96 (0.95–0.97)	<0.001
Fatigue Severity Scale, mean ± SD	4.19 ± 1.57	0.627	491.36	0.261	1.30 (1.22–1.38)	<0.001
Lupus (SLE)	($n = 73$)					
Pain score, median	5	0.328	440.46	0.078	1.08 (1.05–1.11)	<0.001
PSQI, mean ± SD	8.99 ± 2.97	0.081	463.29	0.037	1.04 (1.01–1.07)	0.012
Relationship Assessment Scale, mean ± SD	25.45 ± 4.19	0.818	370.67	0.014	1.01 (0.99–1.04)	0.252
Fatigue Severity Scale, mean ± SD	4.26 ± 1.38	0.551	410.99	0.268	1.31 (1.22–1.41)	<0.001
Depressive disorder	($n = 32$)					
Pain score, median	5.5	0.600	185.10	0.132	1.14 (1.09–1.20)	<0.001
PSQI, mean ± SD	8.48 ± 2.61	0.782	173.46	0.067	1.07 (1.02–1.12)	0.008
Relationship Assessment Scale, mean ± SD	22.52 ± 7.21	0.983	129.17	0.012	1.01 (0.99–1.03)	0.250
Fatigue Severity Scale, mean ± SD	3.83 ± 1.49	0.876	158.17	0.216	1.24 (1.13–1.36)	<0.001
Healthy controls	($n = 22$)					
Pain score, median	0	0.331	112.07	−17.810	0 (0–0)	0.993
PSQI, mean ± SD	5.18 ± 2.36	0.136	117.70	0.079	1.08 (0.99–1.18)	0.072
Relationship Assessment Scale, mean ± SD	28.06 ± 4.46	0.917	81.43	−0.066	0.94 (0.89–0.98)	0.009
Fatigue Severity Scale, mean ± SD	2.78 ± 1.05	0.311	112.71	0.291	1.39 (1.10–1.65)	0.005

Table 7: Multivariate Model for RA subject cohort.

Variables	Coefficient	Odds ratio (95% CI)	p value
Sex	0.164	1.18 (0.81–1.75)	0.401
Age (years)	−0.001	1.00 (0.98–1.02)	0.970
Education (years)	−0.071	0.93 (0.82–1.05)	0.273
Marital status	−0.016	0.98 (0.82–1.16)	0.854
Physical activity	−0.808	0.45 (0.25–0.74)	0.003
Relationship Assessment Scale	−0.041	0.96 (0.94–0.98)	< 0.001
Fatigue Severity Scale	0.206	1.23 (1.10–1.37)	< 0.001
Model fit summary		Pseudo R^2	*AICc*
		0.999	247.280

rheumatoid arthritis, but whereas higher IL-10 levels correlate with depression in RA, lower levels correlate with depression in lupus. Since IL-10 is normally thought of as an anti-inflammatory cytokine, the association between IL-10 levels and depressive symptoms in RA is unexpected. It may be that increased IL-10 is serving as a proxy for increased inflammation. In this case, increased disease activity produces an increase in IL-10 as the normal immune regulatory systems attempt to damp down the inflammation. IL-10 seems to act differently in lupus and rheumatoid arthritis, being disease suppressive in RA and disease promoting in lupus [24], which may help to explain the contrasting results observed in this study.

IL-6 is also likely to be involved in depression. Treatment with the IL-6 inhibitor Tocilizumab was correlated with decreased depressive symptoms in the RA patients. Furthermore, IL-6 concentration correlated with active depression in the primary depression patients. This supports other studies that have suggested a role for IL-6 in depression [25, 26].

The relatively small number of patients in the control groups and the demographic differences between groups are the principal limitation of the present study. Although the longitudinal design of the study could be a strength, the short time between evaluations represents another limitation.

The use of biological therapeutics is a powerful tool not only for treatment but also for aid in the understanding of the mechanisms and pathogenesis of rheumatic diseases. The ability of these medications to inhibit cytokines or pathogenic cells provides a mechanism to examine the function of these factors in a very specific way, as was done with IL-6 in this work. Experiments are currently underway to examine

the roles of cytokines in RA in an extended longitudinal analysis by examining patients on biologicals. These techniques are capable of being applied to any of the many diseases that are treated with biological therapeutics.

Data Availability

All data used in the generation of this article are available upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported by Portugal Fulbright Commission (Dr. Brian D. Poole).

Supplementary Materials

Supplementary 1. The post hoc power analysis of this project is included as Supplementary Table 1. This shows the likelihood of a type 2 statistical error for each comparison, based on the actual variance observed in our study.

Supplementary 2. The complete database that the study is based on is also included as Supplementary Table 2. This includes the complete data set used for each analysis.

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Clinical Study

Host Immunological Effects of Partial Splenic Embolization in Patients with Liver Cirrhosis

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Purpose. Restoration of the balance between T lymphocyte subsets and between Th1/Th2 cytokines together with improvement of antitumor immunity has been reported after hepatosplenectomy in patients with liver cirrhosis (LC) and hepatocellular carcinoma (HCC). However, the detailed effects of partial splenic embolization (PSE) on host immunity are unknown. Accordingly, this study evaluated host immunity in patients with cirrhosis receiving PSE for thrombocytopenia. *Methods*. Twenty-three adult Japanese patients with cirrhosis and thrombocytopenia underwent PSE using straight coils at our hospital between 2010 and 2015. Blood samples were collected before PSE and 4 weeks after PSE. *Results*. The platelet counts were significantly higher 4 weeks after PSE compared with before PSE. The white blood cell count (neutrophils, lymphocytes, and monocytes) also increased significantly after PSE. Furthermore, Th1 cells and Th2 cells showed a significant increase at 4 weeks after PSE compared with before PSE, although there was no significant change of Treg cells. Moreover, serum levels of TNF-alpha, soluble TNF receptor I, and soluble Fas were significantly increased after PSE. There was no significant change of the Child-Pugh score. *Conclusions*. In patients with cirrhosis and thrombocytopenia, PSE not only promoted the recovery of leukopenia and thrombocytopenia but also induced activation of host immunity.

1. Introduction

Splenectomy is considered to be associated with a potential risk of infection, although it is effective in increasing the platelet count. A meta-analysis of studies involving 19,680 patients who underwent splenectomy demonstrated that the incidence of postsplenectomy sepsis was low among adult patients and a higher mortality rate was only observed among children [1]. Partial splenic embolization (PSE) via catheter intervention is an effective alternative to splenectomy that is less invasive than surgery [2–5]. PSE not only increases the platelet counts but also ameliorates portal hypertension and reduces esophageal varices by decreasing blood flow from the splenic vein [6].

The spleen is the largest lymphoid organ in the body and is important for host defenses against disease-causing organisms. Among multiple roles of the spleen in the immune system, it is critical for T cell function [7–9]. However, splenectomy does not impair T cell function in patients with hepatocellular carcinoma (HCC) and liver cirrhosis but promotes recovery of the balance between T lymphocyte subsets, and Th1/Th2 cytokines improve antitumor immunity [10]. Although some studies have examined differences in immunocompetence after embolization or splenic injury [11–14], it is not clear how PSE influences host immunity in patients with thrombocytopenia. Accordingly, this study was performed to evaluate host immunity in patients with cirrhosis and thrombocytopenia who underwent PSE.

2. Methods

2.1. Patients. Twenty-three adult Japanese patients with cirrhosis and thrombocytopenia underwent PSE with straight coils at our hospital between 2010 and 2015. Blood samples

were collected before PSE and 4 weeks after PSE. Each sample was drawn up into a serum tube and centrifuged at 1800g for 10 min to obtain serum that was stored at −80°C. Because the serum level of vascular endothelial growth factor (VEGF) increases over time due to degranulation of platelets [15], samples were processed within 30 min. Serum concentrations of VEGF were measured in duplicate with an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine Human VEGF Immunoassay; R&D Systems, Minneapolis, MN, USA) by an investigator who was blinded to the clinical information about the patients. The serum level of thrombopoietin (TPO) was also measured by ELISA (Quantikine, R&D Systems, Minneapolis, MN, USA). Written informed consent was obtained from each patient after the complications of PSE were fully explained.

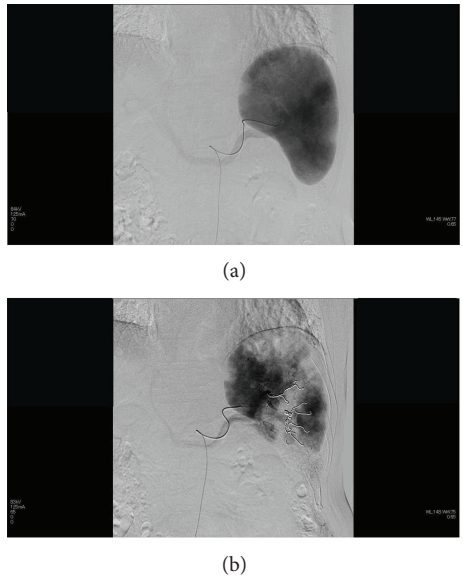
2.2. Partial Splenic Embolization Procedure. A catheter was inserted into the right femoral artery under local anesthesia with 1% lidocaine and was advanced until it reached the hilum of the splenic artery. Then, branches of the splenic artery were embolized by using microcoils and pieces of gelatin sponge, with the objective being to achieve about 60% embolization of the spleen [16] (Figure 1).

2.3. Analysis of CD4-Positive T Cell Subsets. CD4-positive T cell subsets in the peripheral blood were analyzed after nonspecific stimulation with phorbol 12-myristate 13-acetate (PMA), ionomycin, or brefeldin A (Sigma Chemical Co., St. Louis, MO, USA), according to the modified method of Jung et al. [17]. Flow cytometry was used to detect cytoplasmic expression of IFN-gamma and IL-4 by CD4-positive T cells after culture and staining, as reported previously. The percentage of cells producing each cytokine was determined in the total CD4-positive T cell population, which was divided into IFN-gamma-positive/IL-4-negative (Th1) cells and IFN-gamma-negative/IL-4-positive (Th2) cells (Figure 2). In addition, regulatory T cells (Treg cells) were identified as $CD25^{high}/CD127^{low}$ cells (Figure 3).

2.4. Cytokine Assays. The serum level of tumor necrosis factor-alpha (TNF-alpha) was measured in duplicate by using a commercially available enzyme immunoassay (Quantikine, R&D Systems Inc., Minneapolis, USA). The sensitivity of this assay was 2.6 pg/ml, the intra-assay coefficient of variation was ±8.8%, and the interassay coefficient of variation was ±16.7%. In addition, the serum level of soluble TNF-alpha receptor I (sTNFr-I) was quantified by ELISA (Quantikine, R&D Systems Inc.), and the serum level of soluble Fas (sFas) was measured by a sandwich enzyme immunoassay (Quantikine, R&D Systems Inc.). Moreover, sFas ligand (sFas L) was quantified with an immunoassay kit (MBL, Tokyo, Japan). In this immunoassay, concomitant measurement of seven sFas L standards with known concentrations (0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10 ng/ml) was performed together with patient samples and the detection limit for sFas L was <50 pg/ml. Each assay was performed according to the manufacturer's recommendations [18].

2.5. Statistical Analysis. Statistical analysis was done with the Statistical Package for the Social Sciences version 11.0 (SPSS, Chicago, IL, USA). Results are expressed as the mean ± standard deviation (SD). Wilcoxon's signed-rank sum test was used to compare patient characteristics within each group, and a probability of less than 0.05 was considered to indicate statistical significance in all analyses.

This study was approved by the Ethical Review Board of Toho University Medical Center, Omori Hospital (number M17086).



(a)

(b)

Figure 1: Procedure for partial splenic embolization. The catheter was advanced to the hilum of the splenic artery. Branches of splenic arteries were embolized with microcoils and pieces of gelatin sponge, targeting about 60% embolization.

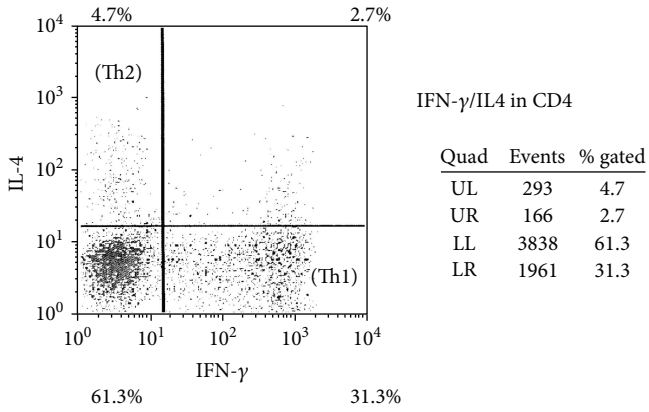


Figure 2: Flow cytometric detection of interferon- (IFN-) γ and interleukin- (IL-) 4 in CD4-positive T cells. Upper left: IFN-γ-negative/IL-4-positive cells (Th2 cells); lower right: IFN-γ-positive/IL-4-negative cells (Th1 cells); upper right: IFN-γ/IL-4 double positive cells (Th0 cells).

3. Results

Twenty-three adult Japanese patients with thrombocytopenia underwent PSE at our hospital between 2010 and 2015.

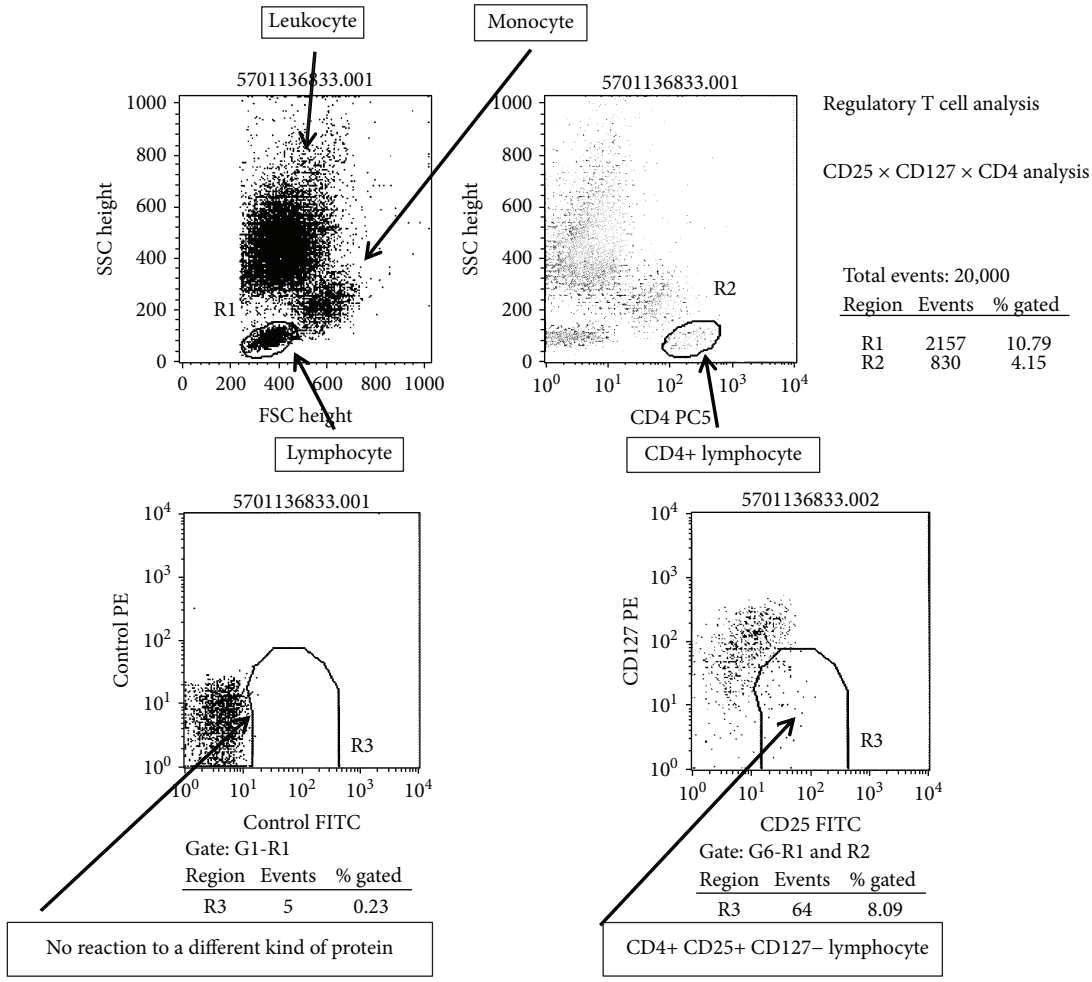


Figure 3: Flow cytometric detection of CD25 FITC and CD127 PE in CD4-positive T cells. Upper left: leukocytes, monocytes, and lymphocytes; upper right: CD4-positive lymphocytes; lower left: no detection of a different protein (control); lower right: CD4-positive/CD127-negative lymphocytes (Treg).

They included 15 men and 8 women aged 37 to 82 years (mean ± SD: 63.2 ± 11 years). One patient had HBV-related LC (B-LC), 14 patients had HCV-related LC (C-LC), 6 patients had non-B-non-C LC, and 2 patients had idiopathic portal hypertension (IPH). Excluding the 2 patients with IPH, the Child-Pugh class was A in 5 patients, B in 13 patients, and C in 3 patients. Twelve patients had HCC, which was stage I in 2 patients, stage III in 2 patients, stage IVA in 6 patients, and stage IVB in 2 patients (Table 1).

3.1. Changes of Parameters after PSE. Table 2 lists various parameters before PSE and 4 weeks after PSE. The white blood cell (WBC) count showed a significant increase after PSE compared to before PSE, with elevation of the neutrophil, lymphocyte, and monocyte counts. The platelet count also increased significantly after PSE. Furthermore, serum levels of VEGF (from 108.8 ± 72 to 426.9 ± 382 pg/ml: $p = 0.0015$, by Wilcoxon's test) and TPO (from 0.69 ± 0.3 to 0.86 ± 0.5 fmol/min: $p = 0.0464$, by Wilcoxon's test) increased after PSE (Figures 4 and 5). Similar changes of these parameters were confirmed in the 21 patients without IPH (data not shown). Liver function (Child-Pugh score) did not improve after PSE, although the prothrombin time increased after embolization.

Table 1: Clinical characteristics for 23 patients.

Number of patients	23
Mean age	63.2 + 11
Gender (M/F)	15/8
Etiology (HBV/HCV/non-B-non-C/IPH)	1/14/6/2
Child-Pugh classification (A/B/C)	5/13/3
With or without HCC	12/9
Stage of HCC (I/II/III/IVA/IVB)	2/0/2/6/2

HCC: hepatocellular carcinoma.

3.2. Changes of Peripheral Blood Th1, Th2, and Treg Cells. Both Th1 cells and Th2 cells showed significant increase after PSE compared to before PSE (Th1 cells: from 22.5 ± 9% to 27.7 ± 12%; Th2 cells: from 3.2 ± 3% to 3.8 ± 3%) ($^*p = 0.0027$ and $^*p = 0.0202$, respectively, by Wilcoxon's

Table 2: Changes of various parameters before and after 4 weeks of PSE.

	Pre-PSE	Post-PSE	p
Ammonia (mg/dl)	82.1 ± 94	51.0 ± 43	*0.1140*
Total bilirubin (g/dl)	1.5 ± 0.9	1.6 ± 1.2	*0.6130*
Direct bilirubin (g/dl)	0.6 ± 0.5	0.7 ± 0.9	*0.2650*
Albumin (mg/dl)	3.2 ± 0.6	3.1 ± 0.6	*0.1050*
AST (IU/l)	52.1 ± 30	58.3 ± 44	*0.3890*
ALT (IU/l)	34.8 ± 27	32.5 ± 28	*0.0890*
Total cholesterol (mg/dl)	130.2 ± 30	127.4 ± 21	*0.9650*
Blood urea nitrogen (mg/dl)	16.6 ± 8	15.0 ± 6	*0.1050*
Creatinine (mg/dl)	0.8 ± 0.3	0.8 ± 0.2	*0.1300*
Prothrombin time (%)	65.8 ± 14	69.7 ± 13	***0.013****
White blood cell (/mm^3)	2945.4 ± 1471	4704.6 ± 1730	***0.001****
Segment (/mm^3)	1791.2 ± 954	2809.3 ± 912	***0.009****
Lymphocyte (/mm^3)	607.2 ± 215	1038.0 ± 451	***0.006****
Monocyte (/mm^3)	245.9 ± 70	440.9 ± 103	***0.001****
Platelets ($\times 10^4$/mm^3)	4.32 ± 1.5	8.95 ± 5.0	***0.001****
AFP (ng/ml)	2561.3 ± 5704	19806.0 ± 39533	*0.0679*
AFP-L3 (%)	19.3 ± 22	26.2 ± 31	*0.1088*
DCP (AU/ml)	325.2 ± 677	364.5 ± 490	*1.0000*
Child-Pugh score	7.6 ± 1.9	7.4 ± 1.9	*0.4174*

PSE: partial splenic embolization; AST: aspartate aminotransferase; ALT: alanine aminotransferase; AFP: alpha-fetoprotein; DCP: des-gamma carboxyprothrombin. *Statistically different compared with Wilcoxon's signed-rank sum test.

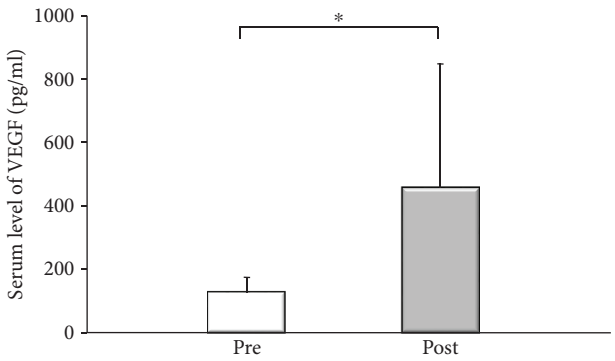


Figure 4: Serum level of vascular endothelial growth factor (VEGF) before and 4 weeks after partial splenic embolization (PSE). Serum VEGF increased significantly after PSE (from 108.8 ± 72 to 426.9 ± 382 pg/ml: $^*p = 0.0015$, by Wilcoxon's test).

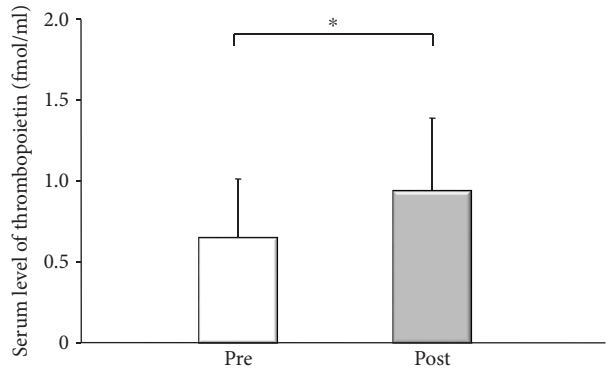


Figure 5: Serum level of thrombopoietin (TPO) before and 4 weeks after partial splenic embolization (PSE). TPO increased after PSE (from 0.69 ± 0.3 to 0.86 ± 0.5 fmol/min: $^*p = 0.0464$, by Wilcoxon's test).

test), although there was no significant changes of Treg cells after PSE (from 9.2 ± 3% to 8.1 ± 2%) (Figure 6). These changes of Th1, Th2, and Treg cells were also confirmed after excluding the patients with IPH (data not shown).

3.3. Changes of Cytokines. The serum levels of TNF-alpha showed significant increase after PSE (from 2.4 ± 2 pg/ml to 3.0 ± 2 pg/ml: $^*p = 0.0077$, by Wilcoxon's test) compared to before PSE, as did the serum level of sTNFr-I (from 1997.3 ± 603 pg/ml to 2392.0 ± 701 pg/ml: $^*p = 0.0022$, by Wilcoxon's test). Serum sFas was also significantly increased after PSE compared to before PSE (from 11.8 ± 3 ng/ml to 13.7 ± 3 ng/ml: $^*p = 0.0076$, by Wilcoxon's test) (Figure 7). The serum levels of sFas L never exceeded 0.15 ng/ml (data not shown). These changes of cytokines were confirmed after excluding the patients with IPH (data not shown).

4. Discussion

In patients with cirrhosis and thrombocytopenia undergoing PSE, this study demonstrated significant elevation of the WBC count after embolization compared to before embolization that reflected an increase in neutrophils, lymphocytes, and monocytes. It is possible that the WBC count increased

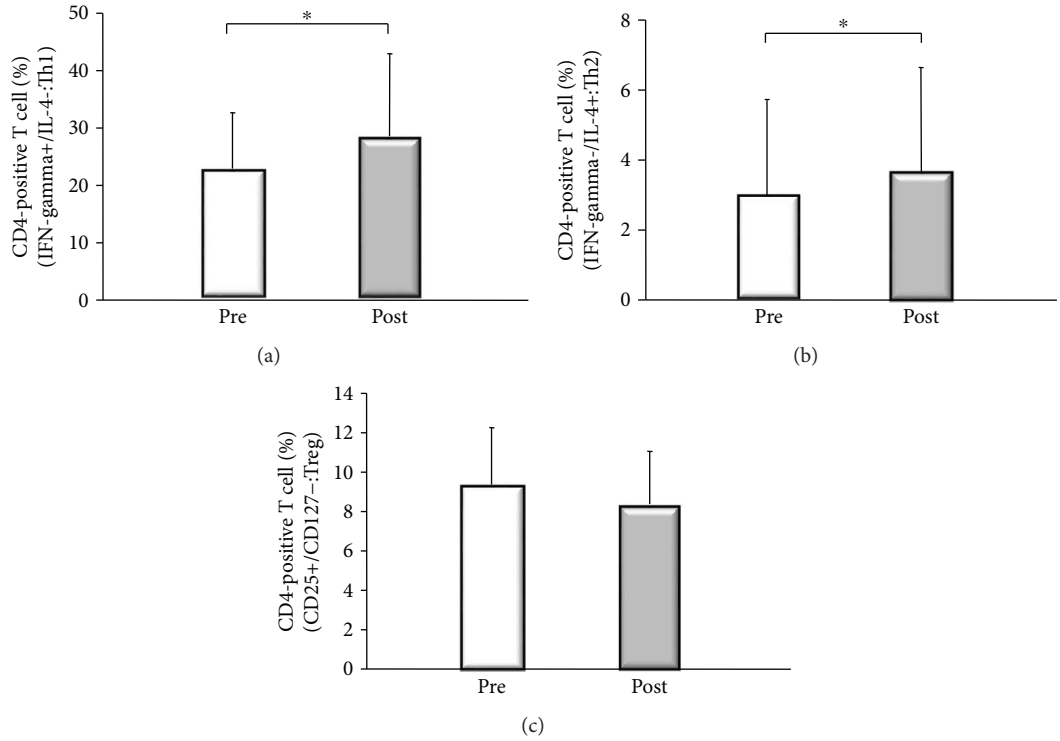


Figure 6: Peripheral blood Th1, Th2, and Treg cells before and 4 weeks after partial splenic embolization (PSE). Upper left: IFN-γ-positive/IL-4-negative cells (Th1 cells) showed a significant increased after PSE compared to before PSE (from $22.5 \pm 9\%$ to $27.7 \pm 12\%$: $^*p = 0.0027$, by Wilcoxon's test). Upper right: IFN-γ-negative/IL-4-positive cells (Th2 cells) also increased significantly after PSE (from $3.2 \pm 3\%$ to $3.8 \pm 3\%$: $^*p = 0.0202$, by Wilcoxon's test). Lower: there was no significant change of regulatory T cells (Treg cells), which were identified as $CD25^{high}$/$CD127^{low}$ cells, between before and after PSE (from $9.2 \pm 3\%$ to $8.1 \pm 2\%$).

due to release of white cells from the spleen. Bone marrow hyperplasia was reported in hypertensive patients with cirrhosis and hypersplenism [19], while serum levels of M-CSF and GM-CSF were significantly reduced by subtotal splenectomy in cirrhosis patients with splenomegaly secondary to portal hypertension [20]. In our patients with cirrhosis and thrombocytopenia who underwent PSE, the WBC count might not only have been increased by release of white cells from spleen but also by normalization of serum M-CSF and GM-CSF levels with suppression of marrow hyperplasia. After investigating the long-term effects of splenectomy in patients with HCV-related LC, Inagaki et al. reported that liver function was likely to be better 5 years after surgery [21]. We did not find improvement of liver function following PSE (evaluated from the Child-Pugh score), although the prothrombin time increased, but a longer observation period may have been needed because our study only assessed change 4 weeks after embolization. We did not assess liver function at 1 year after PSE in the present study, because some of the patients had HCC and received chemotherapy.

We found that the platelet count was significantly higher after PSE, and serum levels of VEGF and TPO increased as well. Splenic pooling of platelets has been shown to cause thrombocytopenia in patients with splenomegaly [22], and the platelet count might have increased due to release of platelets after embolization in our patients. It was reported that serum TPO increases after splenectomy in patients with chronic immune thrombocytopenic purpura [23], and we confirmed that also TPO increases after PSE. TPO is a physiological regulator of megakaryothrombopoiesis [24], and TPO mRNA expression has been detected in the liver and kidneys of humans [25]. Our findings suggested that the evaluation of the platelet count was also induced by increased hepatic production of TPO after PSE. Butthep et al. found the elevation of VEGF in patients with thalassemia after splenectomy [26]. They reported that hypoxia-inducible factor- (HIF-) alpha was a useful biomarker of cellular hypoxia and was correlated with VEGF in patients with thalassemia [27] and that HIF induced transcription of genes ameliorating the effects of hypoxia, including VEGF [28]. These reports support our detection of an increased serum VEGF level at 4 weeks after PSE.

It was reported that Th1 cytokines suppress liver fibrosis, with interferon-γ being a potent inhibitor of the activation of hepatic stellate cells [29, 30], while Th2 cytokines such as IL-4 and IL-3 promote activation of hepatic stellate cells and progression of liver fibrosis [31–33]. Tanabe et al. reported that splenectomy altered the balance of hepatic Th1/Th2 cytokine expression in the direction of Th1 dominance in models of CCl_4-induced and TAA-induced liver fibrosis [34]. In our study, Th1 cells and Th2 cells showed a significant increase after PSE in patients with cirrhosis, with serum levels of TNF-alpha, sTNFr-I, and sFas also increasing significantly. Fas is an important member of a family of receptors that transduce apoptotic signals leading to programmed cell death. It belongs to the tumor necrosis factor

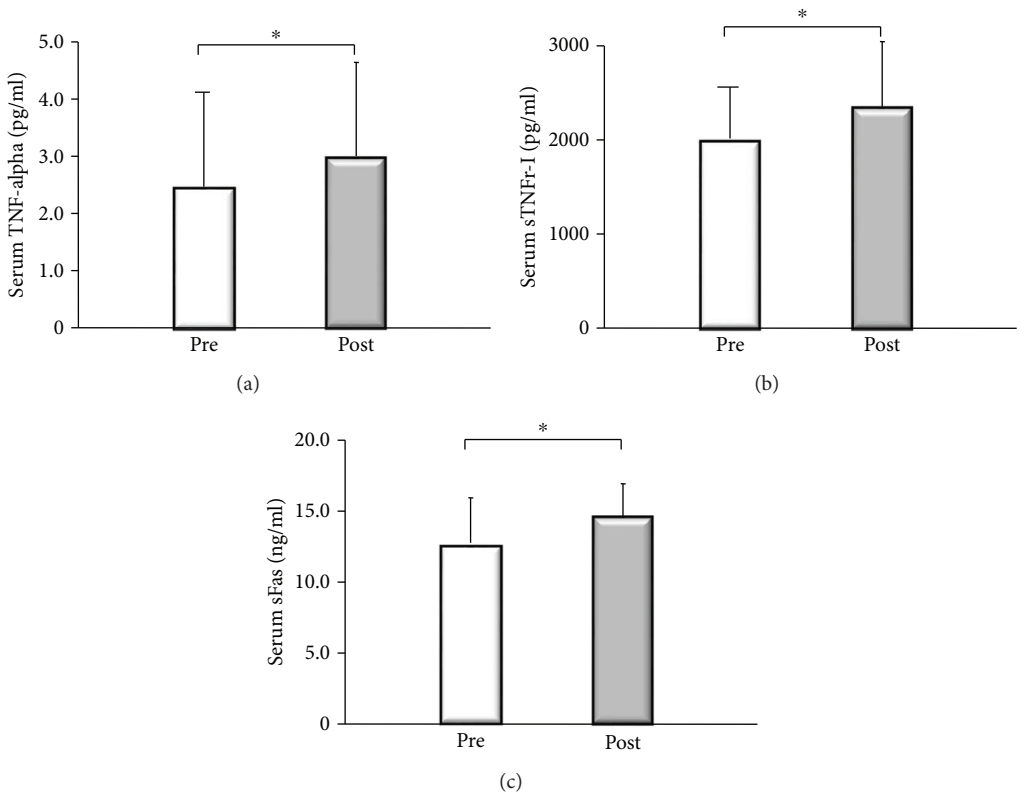


Figure 7: Serum cytokine levels before and 4 weeks after partial splenic embolization (PSE). Upper left: the serum level of TNF-alpha showed a significant increase after PSE compared to before PSE (from 2.4 ± 2 pg/ml to 3.0 ± 2 pg/ml: $^*p = 0.0077$, by Wilcoxon's test). Upper right: the serum level of soluble TNF-alpha receptor I was also increased significantly after PSE compared to before PSE (from 1997.3 ± 603 pg/ml (sTNFr-I) to 2392.0 ± 701 pg/ml: $^*p = 0.0022$, by Wilcoxon's test). Furthermore, the serum level of soluble Fas was significantly higher after PSE than before PSE (from 11.8 ± 3 ng/ml to 13.7 ± 3 ng/ml: $^*p = 0.0076$, by Wilcoxon's test).

(TNF) receptor superfamily, which includes TNF receptor I (TNFr-I) and TNF receptor II (TNFr-II), which were the first members to be discovered and characterized [35, 36]. These receptors are expressed on the surface of various cells, while their soluble forms are released into the circulation after cleavage of the extracytoplasmic domains or alternate splicing [37]. While the serum level of TNF-alpha was increased after PSE in the present study, serum levels of sTNFr-I and sFas were also increased. Thus, the Th1/Th2 balance was not recognized as altered because both Th1 and Th2 increased, but PSE might have improved liver function by inhibiting fibrosis. Moreover, these results indicate that PSE did not cause severe liver injury since the more abundant TNF-alpha or Fas ligand did not bind to TNF-related apoptosis-inducing ligand or Fas. Th1 and Th2 cells cross-regulate their own development. It has been reported that Th2 cytokines inhibit antitumor immunity [38], while activation of Th1 cytokines promotes antitumor immunity [39–42]. We have previously reported that Th1 dominance is lost in HCC patients due to an increase in Th2 cells and that carcinogenesis might be more likely to occur in patients with chronic HCV infection if they show an increase in Th2 cells [43]. Although elevation of Th1 cells after PSE might upregulate antitumor immunity in LC patients with HCC, further investigation will be needed to confirm this. There are two distinct subsets of Treg cells in the peripheral lymphoid organs, which are natural Treg (nTreg) cells that develop in the thymus after recognition of high-affinity autoantigens and induced Treg (iTreg) cells that develop from conventional T cells following peripheral exposure to antigens and cytokines such as TGF-β or IL-10 [44]. These Treg subsets may have synergistic actions or may have different targets that maintain immune homeostasis, although they possibly even have a developmental role [45].

5. Conclusion

In patients with cirrhosis and thrombocytopenia, PSE not only led to improvement of leukopenia and thrombocytopenia but also induced activation of host immunity, although further investigation is needed to confirm these findings.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Poly-L-Arginine Induces Apoptosis of NCI-H292 Cells via ERK1/2 Signaling Pathway

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Cationic protein is a cytotoxic protein secreted by eosinophils and takes part in the damage of airway epithelium in asthma. Poly-L-arginine (PLA), a synthetic cationic protein, is widely used to mimic the biological function of the natural cationic protein in vitro. Previous studies demonstrated the damage of the airway epithelial cells by cationic protein, but the molecular mechanism is unclear. The purpose of this study aimed at exploring whether PLA could induce apoptosis of human airway epithelial cells (NCI-H292) and the underlying mechanism. *Methods*. The morphology of apoptotic cells was observed by transmission electron microscopy. The rate of apoptosis was analyzed by flow cytometry (FCM). The expressions of the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), Bcl-2/Bax, and cleaved caspase-3 were assessed by western blot. *Results*. PLA can induce apoptosis in NCI-H292 cells in a concentration-dependent manner. Moreover, the phosphorylation of the ERK1/2 and the unbalance of Bcl2/Bax, as well as the activation of caspase-3, were involved in the PLA-induced apoptosis. *Conclusions*. PLA can induce the apoptosis in NCI-H292 cells, and this process at least involved the ERK1/2 and mitochondrial pathway. The results could have some indications in revealing the apoptotic damage of the airway epithelial cells. Besides, inhibition of cationic protein-induced apoptotic death in airway epithelial cells could be considered as a potential target of anti-injury or antiremodeling in asthmatics.

1. Introduction

Bronchial asthma is the 14th important chronic disease that affects approximately 3 million patients among the world. Although the mortality of asthma is declining with effective management, patients without effective control still pose a serious financial and social burden on the health care [1]. Many inflammatory cells and cytokines participate in the process of asthma, and eosinophils can be one of the most important cells. The pathophysiological effect of the eosinophils marked infiltration into the airway and the release of toxic protein especially the cationic protein major basic protein (MBP) which can lead to the damage of the bronchial epithelial cells [2, 3].

The quantity and activity of airway epithelial cells are closely related to the severity of asthma especially to airway hyperresponsiveness and airway remodeling. Firstly, airway epithelium forms the first continuous protective barrier against natural pathogenic microorganisms; the damage of the airway epithelium can increase the susceptibility of the mucosal intrinsic and immune cells to antigen which eventually leads to severe airway hyperresponsiveness (AHR) [4]. Besides, in the pathophysiological process of asthma, the activation of proinflammatory cytokines and the damage of the cells let epithelium in a constant state of damage and repair. This could eventually result in a vicious cycle and occurrence of airway remodeling [5]. There has been much circumstantial evidence implicating that

accidents of apoptosis in airway epithelial cells could be an important mechanism which leads to the injury of the epithelial barrier and airway remodeling in asthma, but little is known about the apoptotic damage to the airway epithelium by cationic protein [6–8].

Poly-L-arginine (PLA) is a synthetic cationic polypeptide characterized by its cationic charge with the similar biology and chemical function to MBP and was widely used to mimic the function of MBP in vitro. In the process of eosinophil asthma, with infiltration of eosinophils, cytotoxic cationic proteins were released which finally leads to exfoliation and death of epithelial cells. PLA could stimulate exfoliation and apoptotic death of epithelial cells [9, 10]. However, the underlying molecular mechanism of these cytotoxic cationic proteins to the damage of the airway epithelium is still unclear. In this study, we explored the molecular mechanism of PLA to the damage of the epithelial cells.

2. Materials and Methods

2.1. Materials. PLA was bought from Sigma-Aldrich (St. Louis, MO, USA). And PD98059, an inhibitor of ERK1/2, was also taken from Sigma-Aldrich. PD98059 was dissolved in dimethyl sulfoxide which was purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was from Gibco (Australian origin). Anti-ERK1/2 and anti-phospho-ERK1/2 were from ImmunoWay Biotechnology (Plano, TX, USA). Anti-Bax, anti-Bcl-2, and anti-caspase-3 were from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin was acquired from Zhongshanjinqiao Biotechnology (Beijing, China). Annexin V-FITC Apoptosis Detection Kit with propidium iodide (PI) double staining flow cytometry was purchased from Bestbio Biotechnology Company (Shanghai, China).

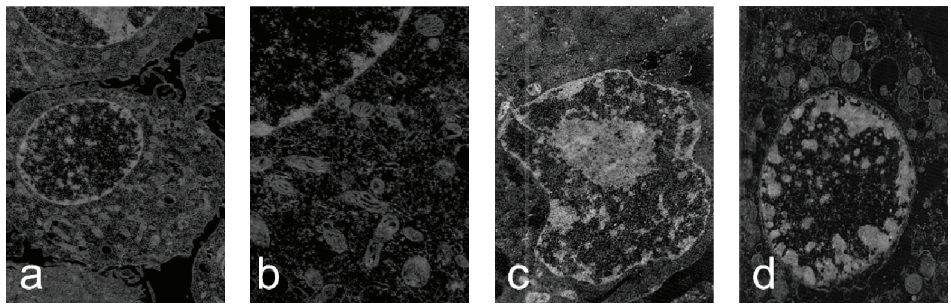
2.2. Cell Culture. NCI-H292 is a kind of human lung mucoepidermoid carcinoma cell line with alveolar type II epithelial cell characteristics. It has monolayer adherent growth status and was from the Chinese Academy of Sciences, Shanghai Institute of Life Sciences Cell Bank. NCI-H292 cells were cultured in RPMI 1640 complete medium (Thermo Fisher Scientific, America) with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 100 μg/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. For experiments, when cells adhere to about 80%, the cells were passaged, digested with trypsin, centrifuged, added 1 ml of culture medium to make cell suspension, then counted under a light microscope by using a cell counter plate (Thermo Fisher Scientific, America), and seeded into six-well plates about 5×10^5 cells per well for cultivation. When the cells in the well converge to 80%, they were treated with PLA at a concentration of 0, 20, 40, or 60 mg/l for 24 h, respectively. In other groups, with previous experiments, the cells were pretreated with the inhibitor of ERK1/2 PD98059 for 30 min.

2.3. Transmission Electron Microscopy. The NCI-H292 cells were treated with 0 or 60 mg/l of PLA for 24 h. Then, the cells were digested with trypsin and centrifuged at 1000 rpm for 3 min. The supernatants were collected. The cell pellets were fixed in 2.5% glutaraldehyde for 5 h. After removing the fixative, each cell mass was washed twice by cold phosphate-buffered saline (PBS), then dehydrated with graded ethanol. After dehydration, each cell mass was immersed with propylene oxide and finally was embedded in epoxy resin. After incubation in citrate for 15 min, thin sections of 50–70 nm thickness were prepared, and the sections were observed directly under transmission electron microscopy (TEM; JEM-1230, Japan).

2.4. Flow Cytometric Analysis. The NCI-H292 cells were digested and made into a suspension, then counted by a cell counter plate under the microscope (TS100, Nikon, Japan) and seeded into a six-well culture plate for 15×10^4 cells per well. The cells were exposed to increasing concentrations of PLA (0, 20, 40, and 60 mg/l), respectively, for 24 h. In another group, the cells were grouped into control, PD98059 (PD), PLA, and PLA + PD groups. With the experiments, the cells in groups PD and PLA + PD were pretreated with PD98059 20 μM for 30 min. After pretreatment with PD98059, 20 mg/l PLA was added into the group of PLA and PLA + PD for 24 h, then the cells were collected into centrifuge tubes, washed with 400 μl annexin V, and suspended for 10–15 min. After staining with 10 μl of PI staining solution, the cells were incubated for 5 min in the laser eight-color flow cytometer, and each tube was shaken evenly. The fluorescence of FITC was detected by a wavelength of 515 nm pass-band filter, and PI was detected by the other filter with a wavelength over 560 nm. Then, the parameters were set and the analysis area was selected as following: the abscissa was set as FITC to represent annexin V-FITC and the vertical coordinate was set as PE-Texas which represents the PI. The cells and apoptotic cells were evaluated by a quadrant gate tool.

For the result determination, the apoptosis rate was analyzed by Flow Jo software (BD FACSVerse, the USA). On the flow cytometer bivariate scatter plot, the normal living cells were on the lower left quadrant which was FITC−/PI−, and the necrotic cells were on the upper left quadrant of which was FITC−/PI+; the lower right quadrant represented the early apoptotic cell which showed FITC+/PI−, and the upper right quadrant was the late apoptotic cells which showed FITC+/PI+.

2.5. Western Blot. The NCI-H292 cells were divided into five groups with concentrations of 0 (control), 10, 20, 40, or 60 mg/l PLA, respectively. Others were divided into the control group, PD group, PLA group, and PLA + PD group. The whole-cell proteins were extracted with RIPA buffer (0.1% SDS, 1% Nonidet P-40, 150 mM NaCl, 0.5% deoxycholic acid, and 50 mM Tris-HCl, pH 7.4) which contained the protease inhibitor phenylmethanesulfonyl fluoride. 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve the total cellular protein, and then the protein was transferred to polyvinylidene fluoride (PVDF) membranes. Next, 5% nonfat milk 2.5 g was used and dissolved in 50 ml Tris-buffered saline with Tween 20 (TBS-T) to block the membranes at room temperature for 2 h. After washing the membranes with TBS-



(a) (b) (c) (d)

Figure 1: Effects of PLA on the morphology of NCI-H292 cells. (a) Control cells were flattened; the cytoplasm stretched to the periphery and adhered to the growth of the support (×8000). (b) In the control group, the mitochondrial ridge displayed continuous integrity and was regularly arranged (×20000). (c) After treatment by PLA, the surface of the nuclear membrane was uneven and the chromosomes were concentrated under it (×8000). (d) After treatment by PLA, the mitochondria were round and empty (×20000).

T three times and 10 min per wash, the membranes were incubated with rabbit anti-human antibodies against ERK1/2, p-ERK1/2, Bcl-2, Bax, and caspase-3 or mouse anti-human β-actin antibody (all at 1 : 500 dilutions), respectively, at 4°C overnight. In the next morning, the membranes were as mentioned before and incubated with the appropriate horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies at a certain degree of dilution (1 : 5000 for ERK1/2, Bax, and caspase-3; 1 : 8000 for p-ERK1/2 and Bcl-2; and 1 : 10000 for β-actin) for 2 h at room temperature. Finally, they were washed with TBS-T, and protein immunostaining was observed by enhanced chemiluminescence (ECL, Thermo Scientific).

2.6. Statistical Processing. All experiments were repeated three times. SPSS version 16.0 (IBM, Armonk, NY, USA) was used to analyze statistics. All values were tested for normality and shown as mean ± standard. For more than two sets of comparisons, one-way analysis of variance (ANOVA) was applied. When assuming that the variance between the two groups is equal, Fisher's least significant difference test (LSD-t) was used, while for not equal, Dunnett's T3 was used. It is statistically different when P values are less than 0.05.

3. Results

3.1. PLA Altered the Morphological Characteristics of NCI-H292 Cells. The morphology of the cells was observed under transmission electron microscopy. In the control group, the morphology of NCI-H292 cells was typical for epithelial cells. The nuclear membrane was complete and the border was clearly visible (Figure 1(a)). The mitochondrial morphology was normal and cristae were arranged regularly (Figure 1(b)). In contrast, cells exposed to 60 mg/l of PLA for 24 h showed typical apoptotic changes in morphology. The nucleus membrane shrank and the chromatin was concentrated under the nuclear membrane (Figure 1(c)). Besides, mitochondria swelled and cristae became disordered and irregular (Figure 1(d)).

3.2. PLA Induces Apoptosis in NCI-H292 Cells in a Concentration-Dependent Manner. Annexin V-FITC/PI double staining assays were used to evaluate the apoptosis induced by PLA following the manufacturer's instructions, which was then measured by flow cytometer. PLA significantly promoted apoptosis of NCI-H292 cells. The apoptosis rates in NCI-H292 cells have increased after treatment of PLA (Figures 2(a)–2(e)). The proportion of annexin V-positive/PI-negative cells increased from 7.82% to 55.23% after being treated with increased doses of PLA for 24 h. Compared with 4.97% in the control group, the differences at each concentration of PLA were statistically significant ($P < 0.001$; Figure 2(f)). In conclusion, the consequences were in agreement with earlier results that the apoptosis happened to cells by PLA. Furthermore, the induction of apoptosis in NCI-H292 by PLA was in a concentration-dependent manner.

3.3. The Effect of PLA to the ERK1/2 Pathway in NCI-H292 Cells. There is much evidence to show that mitogen-activated protein kinases (MAPK) play an important role in cell proliferation, differentiation, and apoptosis [11]. Extracellular signal-regulated kinase (ERK1/2) signaling pathway was the earliest discovered classical signal transduction pathway of Ras-Raf-MAPK involved in the process of cell apoptosis [12]. Our results showed that ERK1/2 can be phosphorylated by PLA. When the concentration of PLA was 10 mg/l, p-ERK/ERK achieved statistical significance. While at a PLA concentration of 20 mg/l, p-ERK/ERK has reached its peak value. ($P < 0.01$; Figures 3(a) and 3(b)).

3.4. The Effects of PLA to the Expressions of Antiapoptosis and Proapoptosis Proteins in NCI-H292 Cells. The molecular mechanism of apoptosis is complicated. The Bcl-2 family includes antiapoptosis protein like Bcl-2 and proapoptosis protein like Bax. The imbalance between the Bcl-2 families can stimulate the release of cytochrome c and ultimately activates caspases such as caspase-3 which eventually promotes apoptotic death of cells [13]. As our results showed (Figure 4), at a PLA concentration of 20 mg/l, the decrease of Bcl-2 presented a statistical difference. At a PLA concentration of 10 mg/l, the increase of Bax and the decrease of Bcl-2/Bax presented a statistical difference. Besides, the expression of caspase-3 increased with an increased concentration

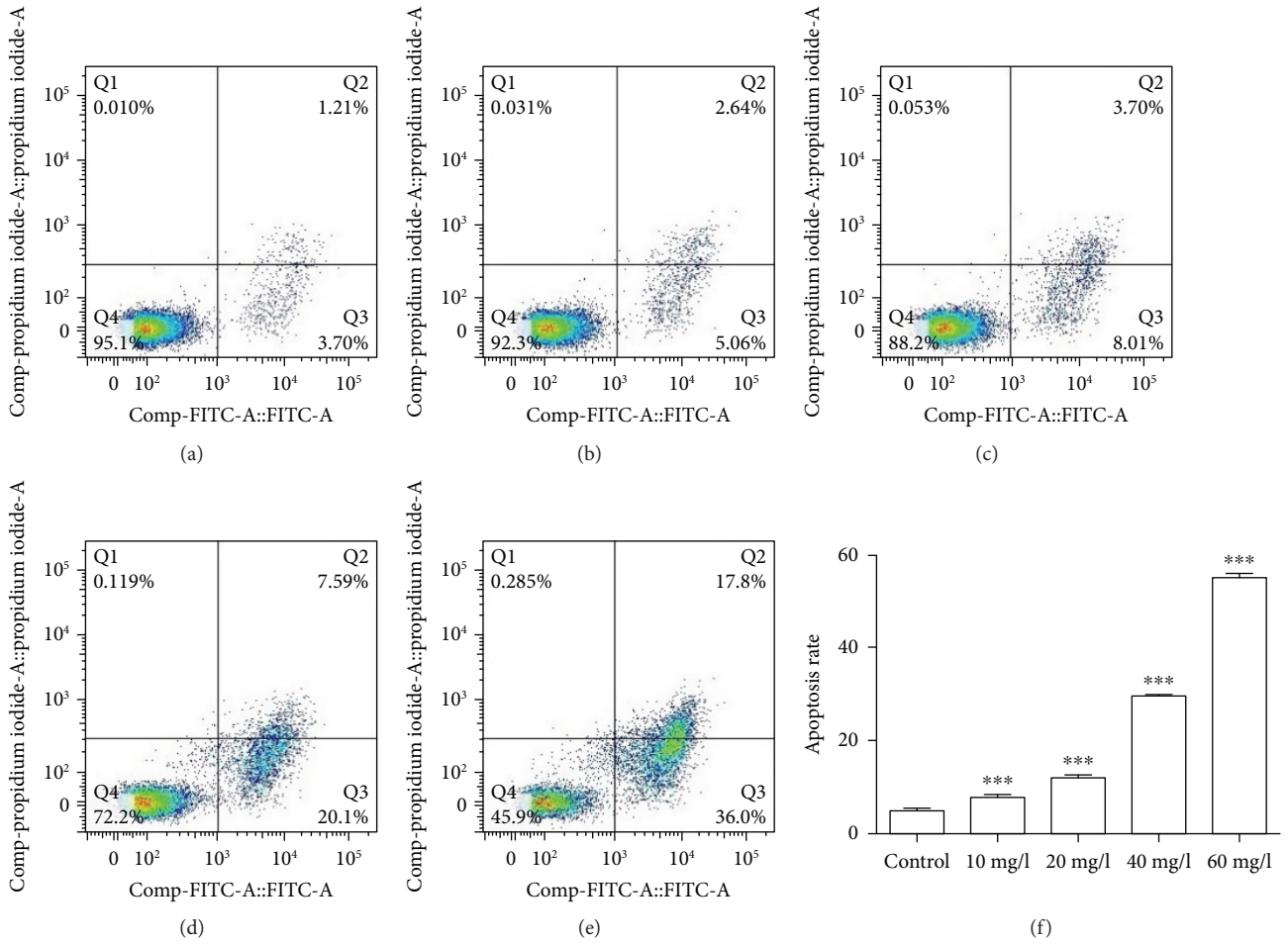


Figure 2: Apoptosis rates in NCI-H292 cells after PLA exposure for 24 h. (a) Control group, (b) 10 mg/l PLA group, (c) 20 mg/l PLA group, (d) 40 mg/l PLA group, and (e) 60 mg/l PLA; (f) the apoptotic rate bar graph. With the increase concentration of PLA, the apoptosis rate of NCI-H292 cells increased. Compared with the control group, $^{***}P < 0.001$.

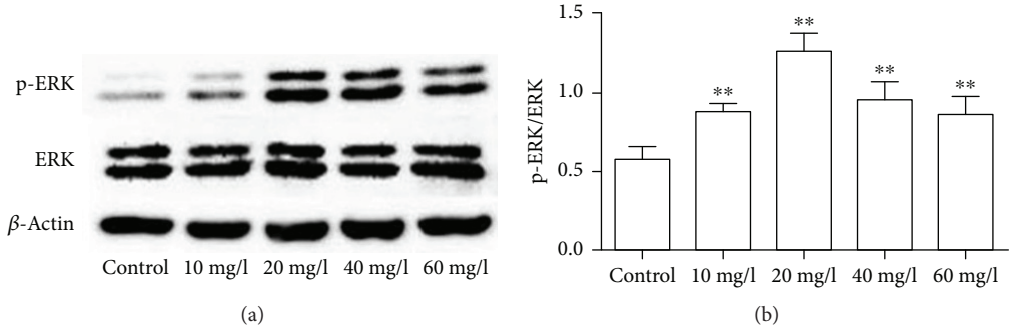


Figure 3: Expression of p-ERK/ERK in NCI-H292 cells. (a) Protein expression bands were detected by western blot. (b) The bar chart shows the ratio of p-ERK/ERK. Compared with the control group, phosphorylation of ERK1/2 increased significantly in groups treated with PLA, especially at a PLA concentration of 20 mg/l. $^{**}P < 0.01$.

of PLA. In conclusion, the balance between proapoptosis protein and antiapoptosis protein was destroyed by PLA in NCI-H292 cells, and this further activated caspase-3.

3.5. PD98059 Inhibits PLA-Induced ERK1/2 Phosphorylation and Apoptosis in NCI-H292 Cells. In order to explore the association between ERK1/2 and apoptosis in PLA-treated cells, we pretreated the cells with PD98059 (20 μM), the specific inhibitor of ERK1/2 [11]. The apoptosis rate of NCI-H292 cells in the PD98059 (20 μM) group was 8.67%, which was significantly lower than the 20.72% apoptosis rate in the PLA group. Besides, PD98059 can inhibit PLA-induced apoptosis in NCI-H292 cells, and the apoptosis rate of NCI-H292 cells decreased and presented statistically

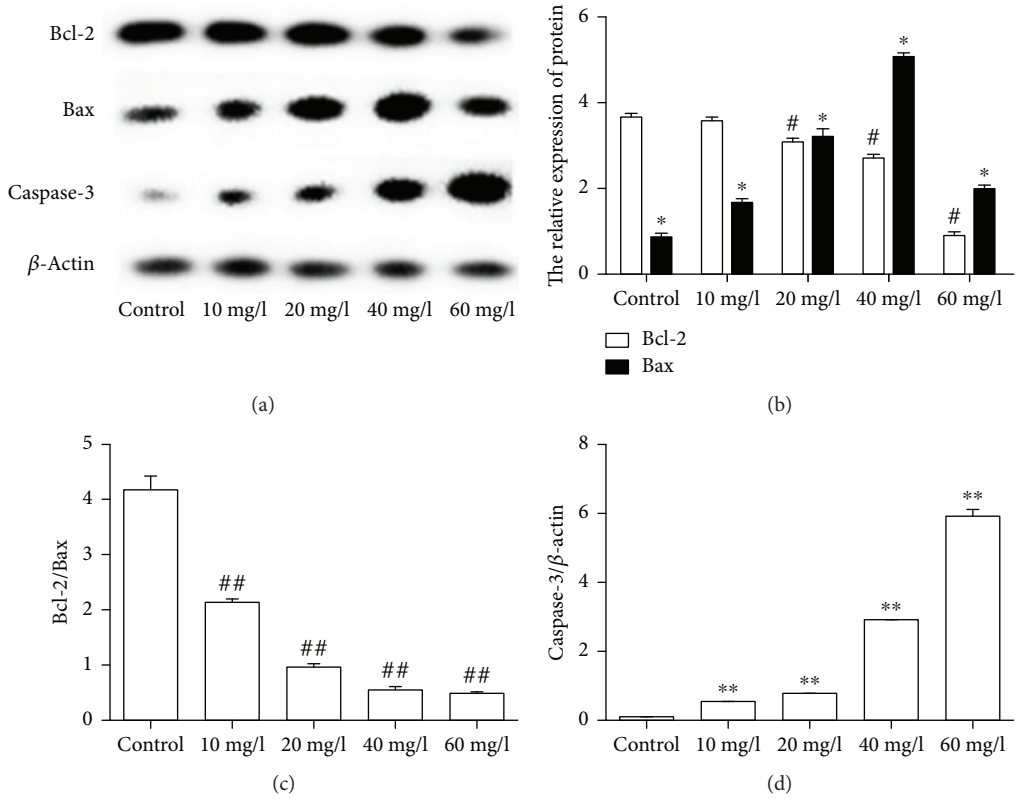


Figure 4: Expression of Bax, Bcl-2, and caspase-3 in NCI-H292 cells. (a) Protein expression bands were detected by western blot. (b) The bar charts of Bax/β-actin and Bcl-2/β-actin. (c) The bar chart shows the ratio of Bcl-2/Bax. (d) Expression of caspase-3 normalized to β-actin. Compared with the control group, the expression of Bcl-2/Bax decreased and cleaved caspase-3 increased. $^{\#}P < 0.01$, $^{\#\#}P < 0.01$, $^{*}P < 0.01$, and $^{**}P < 0.001$.

significant in the PLA + PD group in contrast to the PLA group ($P < 0.001$; Figure 5).

3.6. The Effects of Inhibition of ERK1/2 to the Expressions of Apoptosis-Related Proteins. Since apoptotic cells decreased in the group pretreated with PD98059, we further explored the mechanism of such effects. In contrast with the PLA group, as the phosphorylation of ERK1/2 was inhibited by PD98059, the expression of proapoptotic protein Bax was decreased and the expression of antiapoptotic protein Bcl-2 was restored (Figure 6(a)). And the ratio of Bcl-2/Bax was similar to the blank control group (Figure 6(b)). Besides, the activation of downstream apoptotic molecules caspase-3 was reduced ($P < 0.001$; Figure 6(c)). That is to say, the inhibition of ERK1/2 can decrease PLA-induced apoptosis in NCI-H292 cells.

4. Discussion

The airway epithelial cell as a protective barrier plays an important role in the respiratory system. And the damage of the airway epithelium has been considered as an important etiology of asthma [14]. Major basic protein (MBP) derived from the eosinophils is a natural cationic polypeptide mainly containing arginine and lysine. MBP is considered as one of the markers in eosinophil asthma, and the secretion of MBP contributes to the damage of epithelial cells. Poly-L-arginine (PLA) is widely used to study the function of MBP in vitro. It has already been reported that PLA can induce apoptosis-like changes in airway epithelial cells. PLA can blur the nucleus membrane, resulting in shrinkage and condensation of the nucleus. In our study, we firstly determined that PLA contributes to apoptosis morphology changes in NCI-H292, and this was in consistence with the previous studies [9, 15]. But the potential molecular mechanism seems unclear and few studies have reported it. Hence, we further explored the signal molecules involved in this process.

Mitogen-activated protein kinases (MAPK) are an important signaling pathway in many cell functions. There are more than 20 subtypes of MAPK in mammals and ERK1/2 is one of the most widely studied members. Much evidence has showed the high activation of ERK1/2 in asthma, and the phosphorylation of ERK1/2 has played a role in the activation of apoptosis [16, 17]. It has been reported that the phosphorylation of p38 MAPK is related to the inhibited growth of breast cancer [18].

That is to say, different members of the MAPK family play different roles in cells. And in this study, we found that PLA-induced apoptosis in NCI-H2929 cells was accompanied by the activation of ERK1/2.

Apoptosis acts a pivotal part in maintaining homeostasis between cell survival and death. The permeability of mitochondrial membrane has been shown to play a role in

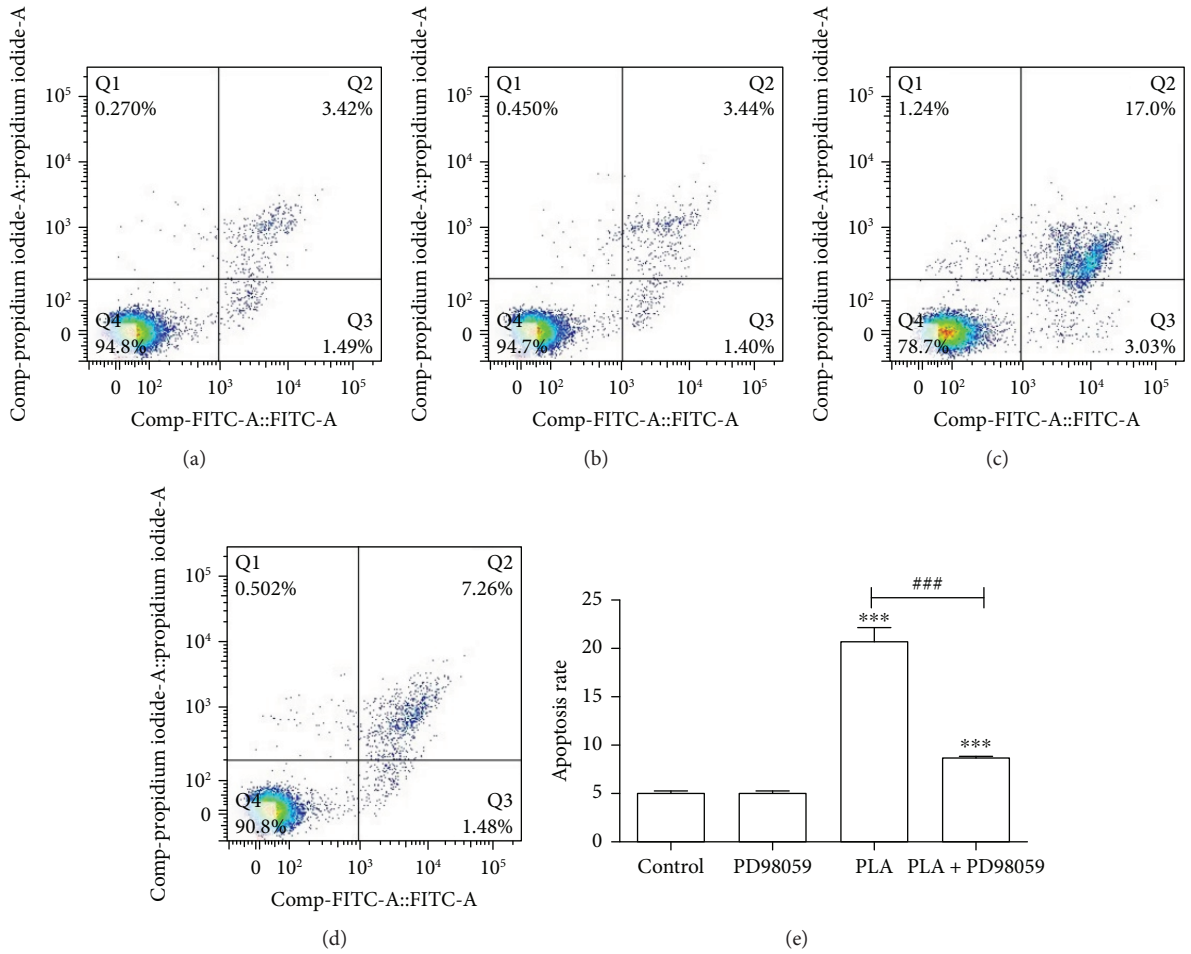


Figure 5: Apoptosis of NCI-H292 cells treated with PLA and PD98059. (a) Control group, (b) PD group, (c) PLA group, and (d) PLA + PD group. (e) The apoptotic rates of the control group, PD group, PLA group, and PLA + PD group. Compared with the control group, apoptosis in NCI-H292 cells was increased by PLA; $^{***}P < 0.001$. Compared with the PLA group, the apoptosis rate decreased in the PLA + PD group; $^{###}P < 0.001$.

apoptosis. Increased mitochondrial membrane permeability eventually leads to the release of cytochrome c and activation of caspase cascades. The Bcl-2 family involves antiapoptotic members like Bcl-2 and Bcl-Xs and proapoptotic members like Bax, Bad, Bak, and Bcl-Xs. They have played a role in sustaining the stability of mitochondrial membrane and the release of cytochrome c [18, 19]. Caspase-3 is called "death-executing protease" which is located in the downstream of Bcl-2 family and occupies a central position of apoptosis [20]. Bax can promote the release of cytochrome c from the mitochondria to the cytosol which eventually contributes to the activation of caspase-3. When Bax is overexpressed in cells, the number of Bax/Bax homodimers increases and cells become more allergic to death signals. While the expression of Bcl-2 increases, the Bax/Bax dimers dissociate in large amounts and produce a more stable Bcl-2/Bax heterodimer to fight against apoptosis so as to prolong cell survival [21, 22]. The imbalance of Bcl-2/Bax leads to many dysfunctions such as the increase of mitochondrial membrane permeability and intracellular calcium as well as oxidative stress. All these can affect the cell's respiratory function and induce cell apoptosis [23]. Therefore, the balance between these two functionally opposite proteins is the key to cell survival [24]. In this study, the decrease of Bcl-2 and the increase of Bax were discovered in groups with PLA especially at a PLA concentration of 40 mg/l. Moreover, the rate of Bcl-2/Bax decreased in a dose-dependent manner. Hence, we postulate that PLA can destroy the balance between Bcl-2 and Bax and further activate caspase-3 cascades. Then, we measured the expression of caspase-3. The results were in consistence with our expectations. The expression of caspase-3 increased in a concentration-dependent manner in groups that were treated with PLA. In order to further verify the apoptosis in NCI-H292 cells induced by PLA, in vitro, we quantitatively analyzed the apoptosis rate by FCM. As the results showed, apoptosis in NCI-H292 cells induced by PLA was in a dose-dependent manner. All these at least could demonstrate that PLA-induced apoptosis in NCI-H292 cells in a concentration-dependent manner, and this process involved the activation of the mitochondrial pathway.

The ERK pathway has been shown to directly affect mitochondrial function by inhibiting mitochondrial respiration [25] and reducing mitochondrial membrane potential [26].

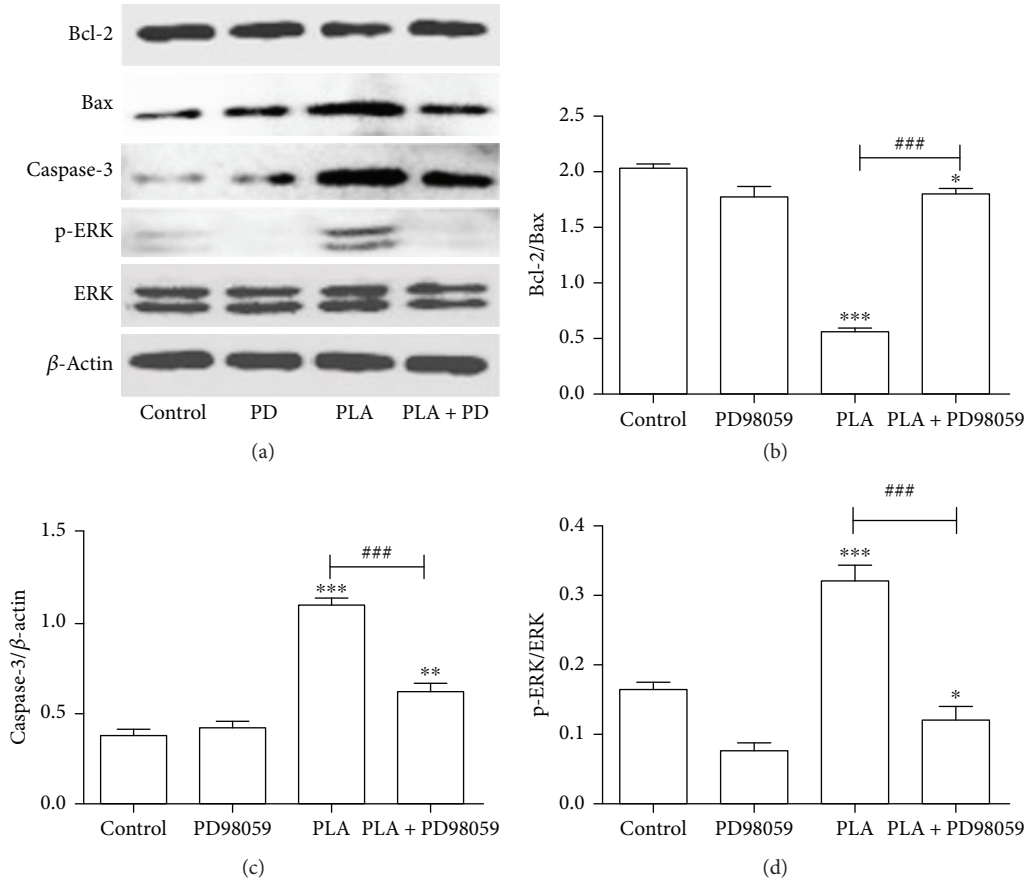


Figure 6: Expression of Bax, Bcl-2, caspase-3, and p-ERK/ERK in NCI-H292 cells treated with PLA and PD98059. (a) Protein expression bands were detected by Western blot. (b) The bar chart of Bcl-2/Bax. (c) The bar chart of caspase-3/β-actin. (d) The bar chart of p-ERK/ERK. Compared with PLA group, PD98059 significantly inhibited the activation of ERK1/2 and also blocked the regulatory effect of PLA on Bcl-2/Bax and cleaved caspase-3. $^{\#\#\#}P < 0.001$. Compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

The phosphorylation of ERK1/2 leads to the disruption of mitochondrial membranes and release of cytochrome c [27, 28]. The activation of ERK1/2 can upregulate a proapoptotic protein, such as Bax [29, 30], and downregulate an antiapoptosis protein such as Bcl-2 [31, 32], which facilitate the release of cytochrome c and apoptosis. But whether the increase of Bax and decrease of Bcl-2 protein in the PLA-treated NCI-H292 cells were through ERK1/2 signal remains unclear, so we inhibited the ERK1/2 by PD98059. In our study, we found that PLA-induced apoptosis in NCI-H2929 cells was accompanied by the activation of ERK1/2. And in order to explore the underlying association between ERK1/2 and apoptosis, we use the inhibitor of ERK1/2 PD98059 to explore the association between ERK1/2 and apoptosis-related protein. As the results showed, the inhibition of ERK1/2 can restore the antiapoptotic protein Bcl-2 and downregulated the proapoptotic protein Bax. All these eventually lead to a reduced expression of the apoptosis protein caspase-3 and decreased apoptosis rate. Hence, we concluded that PLA can activate ERK1/2 to promote apoptosis in NCI-H292 cells, and the inhibition of ERK1/2 by PD98059 can suppress the apoptotic damage in cells, and this could provide a new therapeutic target of epithelial damage in asthmatics.

In summary, PLA can induce apoptosis in NCI-H292 cells in a concentration-dependent manner. And through our study, we could at least conclude that this process involves the activation of ERK1/2 signaling pathway and mitochondrial pathway. The phosphorylation of ERK1/2 leads to the imbalance of the proapoptotic protein like Bax and antiapoptotic protein like Bcl-2. Hence, the ratio of Bcl-2/Bax altered in favor of apoptosis. And this further facilitates the expression of apoptosis protein caspase-3. That is to say, PLA can activate mitochondrial-related apoptosis signaling pathway through ERK1/2 in NCI-H292 cells. In addition, the inhibition of ERK1/2 could reverse the unbalance between the antiapoptotic protein and the proapoptotic protein. However, it is unclear whether other signaling pathways have been involved in PLA-induced apoptosis. Future research could be explored for the mechanism of airway epithelial cell injury induced by PLA in a broader context, which can provide additional solutions for the remission and treatment of eosinophil asthma.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Ya-Ni Wang and Ling-Ling Zhang contributed equally to this work.

Acknowledgments

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Review Article

Adverse Immunologically Mediated Oral Mucosal Reactions to Systemic Medication: Lichenoid Tissue Reaction/Interface Dermatitis-Stomatitis, Autoimmune Vesiculobullous Disease, and IgE-Dependent and Immune Complex Reactions

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Drug-induced hypersensitivity immune reactions are exaggerated immunoinflammatory responses to allergenic components of the medications that occur in genetically susceptible subjects. The type of hypersensitivity immune response generated, whether antibody mediated or T cell mediated, or an immune complex reaction is determined by multiple factors, including the molecular characteristics of the allergen, the route of administration of the medication, the manner of presentation of the allergen by antigen-presenting cells to naïve T cells, the repertoire of the T cell receptors, and the cytokine profile within the microenvironment. This review deals with the clinical and histopathological aspects of adverse immunologically mediated oral mucosal reactions to systemic medication. We elaborate on diseases showing features of lichenoid tissue reaction/interface dermatitis-stomatitis, autoimmune vesiculobullous oral lesions, and immunoglobulin E- (IgE-) and immune complex-mediated oral reactions to drugs.

1. Introduction

Adverse immunologically mediated oral mucosal reactions to systemic medications are not uncommon, are variable in nature, and appear to be genetically determined. Most are mild, but some can be severe and even life threatening; so, prompt diagnosis, immediate withdrawal of the offending drug, and appropriate treatment are crucial [1].

The phenotypic diversity of drug-induced immune hypersensitivity reactions is the outcome of a complex and dynamic pathogenic process. Depending on their molecular concentration and on the context of the microenvironment, different molecular signals can mediate different or sometimes similar immunological effects; and there are interactions between multiple genes, cellular pathways, and cells. The aggregate of this integrated activity is not linear and cannot be derived from summation of the activities of the singular pathways, genes, or cells [2–4].

Susceptibility to adverse drug reactions may be increased by genetic factors determining drug metabolism, such as genetic polymorphism of cytochrome p450 enzymes, drug acetylation and methylation, and the genetic variants determining the type and magnitude of certain immune responses. These determinants include the specific human leukocyte antigen (HLA) haplotype, the T cell receptor (TCR) repertoire, or the toll-like receptor activity [1, 5]. Subjects with vascular collagen diseases, with Epstein–Barr or human immunodeficiency virus (HIV) infections, and recipients of bone marrow grafts are at increased risk of adverse drug reactions, probably because of their related immune suppression or immune dysregulation [1, 6].

Systemic medications may induce different drug-specific immunoinflammatory hypersensitivity responses including

type I immunoglobulin E- (IgE-) mediated, type II IgG-mediated, type III immune complex, and type IV T cell-mediated reactions [1]. Each of these may cause a variety of oral mucosal drug eruptions [7].

In the context of drug-induced allergic reactions, the allergen may be the drug itself, a drug metabolite, a vehicle, or a preservative of the medicine. The allergen functions as a hapten, forming immunological conjugates with tissue proteins, which may then on occasion act as immunogens. In genetically predisposed subjects, allergenic medications may de novo induce immune-mediated oral mucosal diseases, may unmask latent subclinical diseases, or may aggravate the clinical course and manifestations [1, 8].

Pemphigus vulgaris, mucosal pemphigoid, linear IgA disease, lichenoid eruptions, lichen planus, lupus erythematosus, erythema multiforme, Stevens-Johnson syndrome, toxic epidermal necrolysis, and anaphylactic stomatitis are some conditions that can be induced or triggered by certain systemic medications. Therefore, in the process of diagnosing a suspected immune-mediated oral mucosal disease, the possibility of drug involvement as the aetiological factor or as a cofactor should always be considered, particularly in those cases which run an atypical clinical course [1].

Although adverse immunologically mediated oral mucosal reactions to systemic medications are generally considered to be mediated by hyperactive drug-specific T cells, it is possible that adverse drug reactions are not drug specific, but rather the result of hyperactivity of effector cells including T cells, natural killer (NK) cells, NKT cells, dendritic cells, or macrophages or of impaired immune regulatory mechanisms or both, unrelated to a specific drug. Such immune dysregulation may facilitate the development of an adverse immune reaction to a bystander drug [9]. It is also possible that reactivation of latent viruses may trigger an exaggerated virus-specific immune response that can cross-react with a bystander drug, inducing an adverse immunoinflammatory tissue reaction [10–13].

As most drug-induced immune-mediated oral diseases have clinical, histopathological, and immunological features similar to those of idiopathic immune-mediated diseases, it is to be questioned whether in both cases the outcomes are pathologically similar, or whether the drug-induced condition merely mimics the spontaneous idiopathic condition via different immunogenic mechanisms [7, 8]. In some cases, immune-mediated drug reactions resolve after withdrawal of the drug; but in other cases, despite withdrawal of the drug, the condition persists, perhaps supporting the notion of similar but differently induced immunopathogenic mechanisms [8]. The immune-mediated diseases which persist after withdrawal of the suspected causative drug should be treated as being spontaneous idiopathic immune-mediated diseases. The objectives are to relieve symptoms, to promote healing, and to prolong periods of remission [14]. In general, highly potent topical or systemic glucocorticosteroids are the main pharmacological agents of choice, but severe cases of immune-mediated oral diseases may necessitate the use of other agents with immunosuppressive and/or anti-inflammatory properties [15].

Table 1: Predictable and unpredictable reactions to systemic medications: general considerations [19].

Predictable drug effects	Unpredictable drug reactions
(i) Expected pharmacological action	(i) Drug intolerance[X]
(ii) Unavoidable side effects	(ii) Idiosyncracy[Y]
(iii) Drug toxicity	(iii) Immunologically mediated
(iv) Adverse effects of known drug-drug interactions	

[X]Known drug reactions occurring at much lower doses or blood concentration. [Y]Unexpected drug reactions which do not occur in the vast majority of subjects taking this particular medication.

When evaluating a patient with a putatively immune-mediated oral mucosal disease who is also taking systemic medications, the question is whether the condition is idiopathic or drug related. To complicate matters, older subjects are often taking several drugs, each of which may be inducing an immune reaction, which may be affected by drug interaction, and changes in the drug regimen create uncertainty as to whether currently or previously used drugs may be implicated. Therefore, reaching a conclusion about whether an immune-mediated reaction is drug-induced or is idiopathic can be difficult if not impossible [12, 13, 16, 17]. Yet another complication is the possibility of exposure to industrial, occupational, or even household agents that can induce or trigger immune responses, thus influencing the pathogenesis and course of immune-mediated oral conditions [18].

Tissue reaction to systemic medications may or may not be predictable (Table 1) [19], but this review deals only with the clinical aspects and the pathogenesis of immunologically mediated adverse oral mucosal reactions to systemic medications.

2. Allergic Sensitization to Drugs

Only a small proportion of subjects who are genetically susceptible will develop adverse drug-induced, immunologically mediated oral mucosal reactions to systemic medications. Sensitization to an allergenic drug is necessary for the generation of T cell-mediated and antibody-mediated allergic immune reactions. The sensitization cascade starts with the detection and processing of the allergen by antigen-presenting cells of the myeloid lineage and is then followed by the presentation of the allergen to naïve T cells in the regional lymph nodes in the context of major histocompatibility complex (MHC) molecules. Depending on the molecular characteristics of the allergen, the route of administration of the medication, the repertoire of the T cell receptors, and the cytokine profile in the microenvironment, the naïve T cells will differentiate into distinct effector T cell subsets, either T helper 1 (Th1), Th2, Th17, or regulatory T cells with their distinct associated cytokine profiles [20, 21]. However, polarized T cell populations maintain functional plasticity with the capacity to produce some cytokines that are not considered lineage specific [22]. Sometimes the drug-

peptide complex may directly activate T cells by interacting with their receptors without prior priming in lymph nodes [1].

In the context of allergic reactions, Th1 polarization with priming of antigen-specific CD4+ T cells and CD8+ cells occurs in the background of Th1 cytokines including interleukin-2 (IL-2) and interferon-γ (IFN-γ), leading to recruitment and activation of eosinophils and monocytes/macrophages that together with the primed T lymphocytes, generate a T cell-mediated delayed immune hypersensitivity reaction [1]. On the other hand, Th2 polarization with the synthesis of allergen-specific IgE by B lymphocytes on the background of Th2 cytokines such as IL-4 and IL-5 leads to the recruitment and sensitization of effector cells including basophils, eosinophils, and mast cells, generating an IgE-mediated immediate immune hypersensitivity response [20].

The various drug-induced, immunologically mediated oral mucosal conditions are phenotypically distinct although their immunopathogenic mechanisms may be the same and are heterogeneous in their genetic determinants, and their aetiopathogenesis is influenced by gene-gene interactions and environmental factors such as infective agents [6, 10]. However, in the case of oral lichenoid tissue reactions such as allergic contact stomatitis, in oral lupus erythematosus or in classical lichen planus, the clinical pictures may be similar despite different pathogenic mechanisms. The same applies to drug-triggered oral autoimmune disorders including the pemphigus and pemphigoid group of diseases or linear IgA disease. The same drug, in the background of different genetic susceptibility or environmental factors may trigger different immune mechanisms, each with its own distinct cytokine profile, resulting in the great diversity of the clinical manifestations [1].

3. Lichenoid Tissue Reactions/ Interface Dermatitis-Stomatitis

A number of clinically diverse immunopathogenic mucocutaneous inflammatory disorders including lichen planus, allergic lichenoid reactions, lichenoid graft versus host disease, lupus erythematosus, fixed drug eruptions, Stevens-Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN), whether idiopathic or induced or triggered by systemic medications, have similar histopathological features. These histopathological features include necrosis, apoptosis, and disorganisation of basal epithelial keratinocytes with cellular changes described as liquefactive/vacuolar associated with a band-like inflammatory cell infiltrate at the dermal/epidermal interface [23, 24] comprising mononuclear inflammatory cells, predominantly activated T lymphocytes but also macrophages and dendritic cells, and less common neutrophils, eosinophils, and natural killer cells. The infiltrating lymphocytes are in many cases so numerous as to obscure the epithelial-connective tissue junction and are thought to directly cause the epithelial damage [23, 25]. All mucocutaneous diseases with the clinical features of lichenoid tissue reactions and the histopathological features described above are termed "lichenoid tissue reaction/interface dermatitis," and to the opinion of the authors, "stomatitis" can be appended [23, 24].

It appears that type 1 IFN-α/β secreted by plasmacytoid dendritic cells within the inflammatory infiltrate of oral lichen planus and cutaneous lupus erythematosus plays an important role in the pathogenesis of these particular diseases. Type 1 IFN mediates Th1-directed immunoinflammatory reactions and recruitment of cytotoxic T cells to the inflamed tissue and upregulate the expression of cytotoxic agents by cytotoxic T cells and NK cells. This amplifies the immunoinflammatory reactions of oral lichen planus and cutaneous lupus erythematosus [26–28].

The degeneration of the basal cell layer of the epithelium in lichenoid tissue reaction/interface dermatitis-stomatitis is a consequence of necrosis of basal keratinocytes characterized by rapid cytoplasmic swelling with breakdown of intracellular organelles and rupture of the cell membrane and/or of apoptosis of basal keratinocytes characterized by chromatin condensation at the nuclear membrane, compaction of intracellular organelles, and cell shrinkage forming apoptotic bodies. This process of cell death directly caused by products of infiltrating lymphocytes blurs the line between the concept of apoptosis and necrosis [23–25, 29, 30].

It has been established that both antigen-specific CD4+ and CD8+ T cells, cytokines and chemokines secreted by local keratinocytes, and dedicated antigen-presenting cells mediate the death of keratinocytes and the related tissue damage. The specific roles that CD4+ T cells and CD8+ T cells play in the pathogenesis of diseases of the lichenoid tissue reaction/interface dermatitis-stomatitis diseases are not well defined, but it is clear that both are essential for the generation of the immune reactions which cause their diverse clinical phenotypes. It appears that lymphocyte-initiated apoptosis of keratinocytes plays an essential role in the pathogenesis of lichenoid tissue reaction/interface dermatitis-stomatitis diseases. In this context, apoptosis may be induced by direct interaction of the cell death receptor Fas/CD95 expressed by keratinocytes and Fas-ligand (Fas L) expressed by effector cells such as T cells and NK cells or indirectly by secretory pathways including release into the microenvironment of tumour necrosis factor-α (TNF-α), soluble Fas L, or cytolytic/cytotoxic granules containing the pore-forming perforin proteins, granulysin, and granzymes which are all members of a family of serine proteases [31–34]. Intracellularly, caspases, a family of cysteine proteinases, ultimately drive the process of apoptosis [30, 34, 35].

Fas-FasL interactions mediated by both antigen-specific CD4+ and CD8+ T cells induce apoptosis of keratinocytes and cytokines secreted by CD4+ T cells including IFN-γ and TNF-α contribute to the tissue damage. It appears that cytotoxic CD8+ T cells containing perforin, granzyme, and granulysin are the principal effectors of death of basal keratinocytes either by apoptosis or by necrosis. Perforin makes pores in the cell membrane of target cells, paving the way for granzyme B containing endosome-like vesicles to enter the target cells, and subsequent granzyme release degrades DNA molecules and induces apoptosis. NK cell-mediated cytotoxicity and NKT cell responses also contribute to tissue

damage in some of the lichenoid tissue reaction/interface dermatitis-stomatitis diseases [24, 25, 30, 33, 35–37].

Th17 cells with their associated IL-17 and IL-22 cytokines may play a role in the pathogenesis of some lichenoid tissue reaction/interface dermatitis-stomatitis diseases, and CD4+ T regulatory cells are important in regulating the CD4+ and the CD8+ T cell immunoinflammatory responses [24].

Antigen-bearing keratinocytes in the basal/parabasal cell layers of the epithelium are thought to be the target of the immune reaction in lichenoid tissue reaction interface dermatitis-stomatitis but the nature of the target molecule is not known. This might be a self-antigen such as Ro/SSA or La/SSB as in lupus erythematosus or a bioactive drug molecule which serves as a hapten to form immunogenic conjugates with self-proteins or even an environmental element such as an infective agent mimicking the molecular structure of a self-antigen and generating a cross-reactive immune response [23, 24]. Regardless of the precise immune mechanism involved, it is clear that imbalances in the immune responses caused either by hyperactive effector immunocytes or by reduced functional activity or number of regulatory T cells, or both, result in the development of adverse drug reaction [9]. Indeed, it has been demonstrated that in lupus erythematosus and in SJS/TEN, the immune regulatory mechanisms are impaired, and consequently, the function of hyperactive T cells is uncontrolled; so, a severe immunoinflammatory reaction to medication may develop [9, 28].

3.1. Fixed Drug Reactions. Following a primary episode of allergic skin/mucosal eruption induced by a drug-specific T cell-mediated hypersensitivity immune reaction in response to exposure to a systemic drug, reexposure to the same or to a chemically closely related drug may induce a recurrent eruption at the same site. This "fixed drug eruption" is probably because CD8+ memory T cells persist at the site of the initial eruption triggering further eruptions on subsequent exposures [38]. These memory cells involved in the pathogenesis of fixed drug eruptions may well be resident lymphocytes involved in controlling latent human herpes virus infection [23, 38]. A fixed drug-induced reaction of the oral mucosa is thus an immunoinflammatory condition clinically manifesting as zones of erythema and oedema, which may progress to erosions or vesicles. The labial mucosa is the oral site most frequently affected [7].

3.2. Lichen Planus/Lichenoid Reaction. Oral lichen planus is an idiopathic immunoinflammatory mucocutaneous condition, but sometimes, in genetically susceptible subjects, it may be triggered by certain systemic medications in which case it is termed lichenoid drug eruption or drug-induced lichen planus. Before a lichenoid drug eruption occurs, there is usually an unpredictable delay varying from a few days to several years of use of a particular drug and once it has developed, the disease may persist long after the drug is discontinued. Idiopathic oral lichen planus and drug-induced oral lichen planus/lichenoid reaction are clinically and histopathologically similar, and lichenoid drug eruptions which mimic lichen planus may even appear bilaterally further increasing the resemblance to lichen planus [16, 17, 39, 40].

Lichenoid drug eruptions are usually ill-defined erythematous erosive lesions with a lichen-like hyperkeratosis, and in both lichen planus and lichenoid tissue reaction, the cytotoxic CD8+ T cells outnumber the CD4+ T helper cells on the background of a Th1 cytokine milieu [23]. Direct immunofluorescence studies show deposits of fibrinogen, fibrin, C3, and sometimes IgM at the basement membrane zone [41].

It is possible that in drug-induced lichen planus/lichenoid reaction, CD8+ T cells may be activated by a drug molecule expressed by basal keratinocytes in association with MHC class I molecules and that the activated cytotoxic CD8+ T cells may trigger necrosis or apoptosis of keratinocytes via the Fas-Fas L, granulysin, or perforin/granzyme pathways [23, 42, 43]. One cannot discard the idea that in what appears to be a drug-induced lichenoid reaction, the drug may in fact have triggered an immunoinflammatory response that then unmasks preexisting subclinical oral lichen planus [44].

3.3. Lupus Erythematosus. Cutaneous lupus erythematosus is a chronic mucocutaneous immune-mediated inflammatory disorder brought about by complex interactions between intrinsic factors including susceptible genes determining immune responses and clearance of apoptotic cells on the one hand and extrinsic factors including ultraviolet radiation, infectious agents, and certain drugs on the other hand. Increased apoptosis, impaired clearance of apoptotic cells, impaired immune regulatory functions with consequent lower activation thresholds of T and B lymphocytes, reactive antibodies against intracellular constituents of nucleosomal DNA proteins and ribonucleoproteins, and dysregulation of the cytokine network, particularly the overexpression of TNF-α and type 1 interferons, all together drive the inflammatory process causing the tissue damage in lupus erythematosus [27, 28, 45].

Upregulation of expression of CD1d receptor by keratinocytes and by antigen presenting cells secondary to epithelial injury, or to inflammation at the basement membrane zone, may cause inactivation of invariant natural killer T (iNKT) cells by CD1d-bound glycolipid antigens. iNKT cells are a functionally versatile subset of T lymphocytes which by secreting into the local microenvironment, a variety of soluble biological factors, and by directly interacting with various adaptive or innate cells, has the capacity either to enhance or to regulate immunoinflammatory processes [46–48]. Furthermore, iNKT cells which also express perforin, granzyme B, and Fas L are believed to play a role in the pathogenesis of cutaneous lupus erythematosus [46, 47].

Lesions of oral lupus erythematosus manifest as well-defined atrophic, erosive, or ulcerated areas with radiating keratotic striae and surrounding telangiectasia, predominantly affecting the buccal mucosa, but also the gingiva, labial mucosa, and the vermilion border of the lip. Although discoid lupus erythematosus may affect only the oral mucosa, it usually occurs together with skin lesions [27].

The mechanisms by which drugs may trigger lupus erythematosus are not well understood, but it has been suggested that certain drugs induce the production of the cytokines TNF-α and IFN-α which have the potential to stimulate and to increase the autoreactive capacity of B and T lymphocytes, or alternatively, some drugs by promoting epigenetic modifications can dysregulate T lymphocyte gene expression, resulting in the T cells becoming autoreactive [27, 28].

3.4. Erythema Multiforme. Erythema multiforme is an acute immune-mediated mucocutaneous blistering disease. The oral mucosa is involved in up to 70% of cases and not infrequently may be the only site affected. Periods of disease activity usually last 10–20 days; remissions last months to several years. On average, within a period of 10 years, there are six episodes of disease activity [49].

Oral erythema multiforme has a predilection for the lips and the anterior part of the mouth but can affect any part of the nonkeratinized oral mucosa. The primary erythematous macules rapidly become blisters and soon rupture leaving painful diffuse multifocal erosions or superficial ulcers surrounded by zones of erythema. The lips are invariably hyperaemic, eroded or ulcerated, split, bleeding, and crusted. There is interference with eating, swallowing, and speech [49–52].

Erythema multiforme may be idiopathic, but most cases are associated with herpes simplex virus or *Mycoplasma pneumoniae* infections or with any one of a variety of drugs [52]. In susceptible subjects, drug-induced oral erythema multiforme usually occurs within a few days of starting a systemic medication and resolves upon its discontinuation [53]. It is likely that a cytotoxic CD8+ T cell immune reaction to a drug metabolite within the oral epithelium plays a fundamental role in the initiation of drug-induced oral erythema multiforme [50].

3.5. Stevens-Johnson Syndrome (SJS) and Toxic Epidermal Necrolysis (TEN). The current literature distinguishes between SJS and TEN but nevertheless draws attention to the similarities and to their often-overlapping presentation. SJS and TEN are related life-threatening immune-mediated conditions characterized by epithelial blisters, which progress to severe, diffuse mucocutaneous erosions with diffuse detachments of necrotic epithelium accompanied by fever and sometimes by toxic visceral effects. In SJS, there is less than 10% of the skin detachment and in TEN, more than 30%, and when there is between 10% and 30% of skin detachment, the condition is conveniently termed "SJS-TEN overlapping syndrome" [19, 54]. All cases of TEN and about 75% of SJS are induced or triggered by systemic medications within one to eight weeks of introducing the offending drug [19].

Drug-specific cytotoxic T cells reactive to keratinocytes, NK cells, the cytokines TNF-α and IFN-γ, and increased levels of perforin, granzyme B, and granulysin are all found in the lesional blisters of SJS/TEN. This suggests that the necrosis of keratinocytes is mediated predominantly by drug-specific HLA-restricted cytotoxic T lymphocytes via a perforin/granzyme-mediated pathway [1, 19, 31, 33, 52, 55].

In addition to necrosis of keratinocytes, the tissue damage in SJS/TEN is also characterized by widespread keratinocyte apoptosis mediated by interactions between the death receptor Fas and its ligand Fas L and by the cytotoxic protein granulysin secreted by activated T cells and NK cells [19, 32, 33, 52, 54, 55].

4. Drug-Induced IgE-Dependent Immune Hypersensitivity Response

IgE-dependent anaphylactic reactions are caused by biological mediators of inflammation released either by tissue mast cells or by circulating basophils or by both. Mast cells and basophils express the high-affinity Fc receptor for IgE, the FceRI. Binding of allergen-specific IgE to FceRI results in sensitization of the cells enabling effector responses so that subsequent exposure to the specific allergen may result in its crosslinking to IgE molecules bound to FceRI-bearing cells. This interaction results in the almost immediate release of preformed biological mediators such as histamine, leukotrienes, and prostaglandins which drive the IgE-dependent hypersensitive effector reactions [20, 56, 57].

In response to stimulation by allergen-specific IgE, mast cells may also de novo produce and release a variety of cytokines, chemokines, and growth factors which have the capacity to recruit and activate innate immune cells including eosinophils, neutrophils, and basophils. The functional activity of mast cells varies at different anatomical sites and is dictated by the cytokine profile and the cells in the specific local microenvironment [58].

Certain drugs have the capacity to directly trigger degranulation of mast cells/basophils thus bringing about pseudoallergic/anaphylactoid reactions by nonimmunological activation of effector pathways [1]. These pseudoallergic anaphylactoid reactions can occur on first exposure to the drug, developing within minutes [1, 19].

4.1. Drug-Induced Anaphylactic Reactions of the Oral Mucosa and Surrounding Tissues. Angioedema and anaphylactic stomatitis are manifestations of drug-induced, IgE-mediated allergic reactions [7]. Angioedema is a localized cutaneous/mucosal oedematous swelling brought about by a temporary increase in vascular permeability mediated by vasoactive biological agents. Histopathologically, there is a perivascular infiltrate of eosinophils and lynphocytes with, and lymphocytes with an increase in the endothelial intercellular spaces and separation of perivascular collagen bundles. Drug-induced histamine release from mast cells/basophils occurs as a result either of an IgE-dependent allergic reaction or of direct IgE-independent degranulation of these cells resulting in histaminergic angioedema. The face is most commonly affected, and occasionally also the pharynx, larynx, oropharynx, or the oral tissues. Angioedema of the mucosa of the upper aerodigestive tract may be life threatening because of the risk of upper airway obstruction [59].

5. Immune Complex-Mediated Hypersensitivity Reaction

Antigen-antibody complexes cause tissue damage by eliciting inflammation at the sites of their deposition. The pathogenesis of this inflammation occurs in three phases: firstly, the formation of antibodies against either endogenous- or exogenous-free circulating antigens and formation of antigen-antibody complexes; secondly, deposition of the complexes in vessel walls with fixation of complement which together with Fc receptors initiates leukocyte recruitment and activation; and thirdly, acute inflammation with tissue damage. The principal morphological manifestation of immune complex disease is acute necrotizing vasculitis with fibrinoid necrosis of the vessel wall and an intense infiltrate of neutrophils extending through the vessel wall into the perivascular zone, where they degenerate into nuclear dust. In the skin, this is referred to as leukocytoclastic vasculitis [60].

Although immune complex disease can be systemic, involving many organs, in the context of this review, we deal with localized deposition of immune complexes in cutaneous and mucosal vasculature. Immunoglobulins and complement components can only be demonstrated in early lesions; but in up to 40% of cases, the causative immune complex cannot be identified. In most cases, the lesions are induced either by drugs or by infective agents [1]. In the classic form of drug-induced immune complex reaction, there is deposition of immune complexes with the activation of complement, resulting in fever, arthritis, oedema, or skin and mucosal lesions. Typically, complement-mediated immune complex tissue damage develops some six days after exposure to the allergen, this being a period required for the production of the drug-specific antibodies [1]. Drug-based immune complexes may activate plasmacytoid dendritic cells expressing Fc receptors which then secrete INF-α, contributing to the adverse immunoinflammatory drug reaction [61].

In the oral mucosa, minor and major aphthous ulcers, aphthous-like Behcet's disease, and erythema multiforme are said to be examples of immune complex hypersensitivity, but while perivascular lymphocytic cuffing and neutrophilic infiltration of the epithelium have been sometimes described, true vasculitis and immune complexes are hardly ever seen [60, 62].

6. Autoimmune Vesiculobullous Diseases

Pemphigus and pemphigoid groups of diseases and linear IgA disease are autoimmune mucocutaneous vesiculobullous conditions in which the oral mucosa is not infrequently affected. These diseases may either be idiopathic or be triggered by a variety of extrinsic agents such as viruses or medications and are clinically characterized by inflammation, blisters, or erosions [7, 10].

It is possible that genetically determined potential autoimmune mucocutaneous vesiculobullous diseases that have been suppressed by intrinsic factors can be triggered by drugs that destabilise the immune network in such a way that the subclinical disease becomes overt [10]. Some allergenic drugs may bond to epithelial or basement membrane zone proteins either to change their antigenicity or to form a hapten-peptide complex, eliciting in a genetically predisposed subject, an immune hypersensitivity reaction, bringing about blisters of the oral mucosa [18, 63, 64]. Another possibility is that drug-induced immunoinflammatory reactions cause local immune dysregulation which in turn leads to loss of control of viral replication and reactivation of latent viruses. In response to the subclinical viral infection, there is upregulation of cytokine expression promoting the development of autoimmune mucocutaneous blistering diseases owing to loss of immune tolerance with increased autoreactive capacity of immune cells to self-antigens [10].

Certain drugs, by nonimmunological mechanisms, can cause epithelial damage by dysregulating the activity of enzymes essential for the function of keratinocytes and for keratinocyte adhesion to one another and to the basal lamina. Drugs containing thiol groups have the biochemical capacity to cause intraepithelial and epithelial-laminal propia separation with the development of oral mucosal blisters [12, 18, 63, 65]. Furthermore, in susceptible subjects, some drugs can stimulate keratinocytes to produce and release cytokines such as TNF-α and IL-1α which can also result in acantholysis [12].

It has been suggested that in the category of drug-induced immune-mediated mucocutaneous diseases, there are two possible responses: one is self-limiting and develops some time after starting the medication and resolving when the drug is withdrawn and the other is triggered rather than induced by a drug and runs a persistent clinical course despite the withdrawal of the offending drug [64, 65].

The clinical, histopathological, and immunological features of idiopathic and drug-induced autoimmune-mediated mucocutaneous blistering diseases are indistinguishable [63, 66]. However, it appears that drug-induced mucosal blistering diseases usually affect younger subjects, undergo remission after withdrawing the offending drug, and respond more favourably to treatment than do idiopathic blistering diseases [63, 64]. Furthermore, it has been reported that subjects with drug-related mucocutaneous blistering diseases less frequently show circulating autoantibodies to self-antigens than do subjects with idiopathic mucocutaneous blistering diseases [18].

6.1. Pemphigus Vulgaris. Pemphigus vulgaris is a potentially fatal autoimmune mucocutaneous blistering disease. The oral mucosa is commonly involved and occasionally is the first site of presentation. The disease runs a prolonged course, with the blisters rupturing soon after formation leaving ill-defined, irregularly shaped, painful superficial erosions or ulcers with marginal tags of epithelium that can be peeled off beyond the margins of the lesion. The lesions are slow to heal, interfering with eating, drinking, swallowing, and speech, with continual formation of new blisters [67, 68].

In pemphigus vulgaris, there is binding of autoreactive IgG to desmosomal proteins (desmogleins) causing the loss of keratinocyte cohesion. This results in acantholysis with the formation of suprabasal intraepithelial blisters with free-floating epithelial cells within the blister (Tzanck cells).

The basal keratinocytes constitute the floor of the blister and do not lose their attachment to the basement membrane zone. Neutrophils and eosinophils may be present in the superficial portion of the lamina propria [67].

It has been reported that up to 50% of first-degree relatives of subjects with overt pemphigus have subclinical immune reactions characteristic of pemphigus, including circulating autoantibodies to desmosomal proteins and epithelium-bound autoantibodies, without having any overt signs of disease [65]. Genetic susceptibility, viral infection, and certain medications are some factors that play roles in the pathogenesis of pemphigus vulgaris and that have the capacity to influence its clinical manifestations and course. It is probable that in subjects with subclinical characteristics of pemphigus, there are some protective genetic factors that prevent the development of the overt disease [65].

6.2. Mucosal Pemphigoid. Mucosal pemphigoid is an autoimmune blistering disease, which often starts in the mouth, and is sometimes limited to the oral mucosa. It is characterized by tissue-bound autoreactive antibodies against structural proteins of the basement membrane zone and of hemidesmosomes that mediate the recruitment of inflammatory and immune cells with the generation of chronic inflammation. This results in the detachment of basal keratinocytes from the underlying basement membrane zone with the formation of subepithelial blisters [69, 70]. Rupture of the blisters leaves painful, irregular, wide-spread superficial ulcers, and the erythematous denuded tissue is covered with a fibrinous pseudomembrane. The buccal mucosa, palate, and the gingiva are the most frequently affected oral sites. The lesions heal without scarring [69].

Characteristic microscopic features of mucosal pemphigoid include a subepithelial chronic inflammatory cell infiltrate of eosinophils, lymphocytes, and neutrophils and a linear deposition of immunoglobulins and complement within the basement membrane zone [69].

Usually, mucosal pemphigoid is idiopathic. However, the configuration of hemidesmosomal and certain basement membrane zone proteins may be altered by drugs or viruses resulting in the development of compound autoimmunogens generating autoimmune reactions. In genetically susceptible subjects, drugs or viruses may also modify preexisting subclinical immune reactions, thus triggering or promoting the development of the disease.

6.3. Linear IgA Disease. Linear IgA disease is an uncommon autoimmune mucocutaneous disorder defined by a linear pattern of IgA deposited in the basement membrane zone. The disease may be idiopathic or drug-induced but the clinical, histopathological, and immunological features are similar in both forms. Oral lesions are not uncommon, manifesting as erythematous macules, subepithelial blisters, or erosions with pain or a burning sensation [71–73].

Histopathological examination shows vacuolar degeneration of basal cell keratinocytes with subepithelial blistering and a predominantly T cell- and neutrophil-mixed cell infiltrate in the superficial portion of the lamina propria. The self-antigens targeted in linear IgA disease are proteins in the lamina lucida and lamina densa of the basement membrane zone, and the tissue damage is caused by T lymphocytes and neutrophils and by cytokines and inflammatory mediators released into the microenvironment. In patients with the idiopathic form of the disease, circulating antigen-specific antibodies may or may not be present but are absent in the drug-induced form [71–73].

In drug-induced linear IgA disease, signs and symptoms usually develop within the first month of using the drug and resolve spontaneously some time after discontinuation of the drug [73].

7. Conclusion

Adverse immunologically mediated oral mucosal reactions to systemic medications are exaggerated immunoinflammatory responses to allergenic components of the medication occurring in genetically susceptible persons. Lichenoid tissue reaction/interface stomatitis diseases may be idiopathic or induced by systemic medications. Antigen-bearing keratinocytes in the basal/parabasal cell layers of the oral epithelium are thought to be the target of the immune reaction in these diseases, but the nature of the target molecules is not clear.

IgE-dependent and immune complex reactions and vesiculobullous diseases are immunopathogenic reactions usually developing when the medication has been used for some time and resolves when it is withdrawn or may be triggered rather than being induced by medication, in which case the disease runs a persistent clinical course despite the withdrawal of the offending drug.

Conflicts of Interest

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

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Research Article

Sample Aging Profoundly Reduces Monocyte Responses in Human Whole Blood Cultures

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Human whole blood cultures are widely used for the investigation of physiological pathways and drug effects *in vitro*. Detailed information on the effect of "sample aging" (the time span between blood collection and experimental start) on the experimental outcome is not readily available in the public domain. We studied the effect of sample aging on the ability of immune cells to respond to cell-specific immune triggers (LPS, PMA/ionomycin, and SEB). Sample aging at room temperature profoundly inhibited the LPS-induced monocytic cytokine release in minimally diluted whole blood cultures. The reduction ranged from 20–50% after 30 minutes to 80–100% after 10 hours and differed between cytokines (IL-1β, IL-2, IL-6, IFNγ, and TNFα). Sample storage at 4°C or 37°C even worsened this. PMA/ionomycin- and SEB-induced cytokine release, both mainly T-cell-driven, were also reduced by sample aging but to a lesser extent (20–50% after 24 hours). Intracellular cytokine staining revealed that the number of LPS-responding cells was not impacted by sample aging and reduced LPS responsivity could also not be explained by apoptosis or downregulated TLR4 expression. Thus, we speculate that sample aging induces an inhibitory pathway downstream from TLR4 in monocytes. These results underline the importance of quick sample handling when investigating innate immune responses in whole blood, especially for monocyte responses.

1. Introduction

Primary human immune cells are widely used for the investigation of physiological pathways and drug effects *in vitro*. Primary cells are often used for research on chronic diseases and for testing new therapies for a wide range of diseases. Such experiments are frequently performed on isolated leukocytes, mostly peripheral blood mononuclear cells (PBMCs). Cryopreserved PBMCs can be analyzed batch-wise, so that samples collected at multiple clinical sites can be analyzed at one central laboratory. Alternatively, *in vitro* experiments can be conducted on whole blood samples, with the obvious benefit that this better resembles *in vivo* conditions [1]. Sample handling is minimal for *in vitro* experiments on whole blood samples: the only variable to control is the time span between blood collection and the start of the experiment. Although most publicly available literature on whole blood-based experiments does not specify this time span, it is evident that the "sample age" may vary substantially between and within experiments, depending on the clinical unit, the laboratory, and the donor population involved. Limited information is available on the effect of the sample age on the outcome of whole blood-based experiments. This is remarkable since a direct relationship between sample age and cell functionality (i.e., cell viability and cell responsiveness to immune triggers) could be expected. In PBMC cultures, apoptosis occurs spontaneously after prolonged sample storage [2, 3]. In whole blood samples, blood settling causes red blood cell and platelet aggregation and alters cell function [4]. Another potential problem in whole blood cultures is the short lifespan of neutrophils [5]. These cells survive for less than 24 hours in the bloodstream and are prone to undergo apoptosis under suboptimal environmental conditions. Apoptotic neutrophils may have secondary effects on other leukocyte subsets in a whole blood-based experiment. To overcome these problems, efforts have been made to preserve whole blood samples for longer periods of time. For example, cryopreservation of the whole

blood has been shown to be feasible for experiments assessing Epstein-Barr virus (EBV) transformation, lymphocyte proliferation, and DNA extraction [6–8]. Freezing of fixed whole blood may also be appropriate for immunophenotyping [9]. However, granulocytes do not stay viable during the freezing process impacting the responses of other cell types [10, 11]. The addition of phytohaemagglutinin (PHA) during sample storage avoided the apoptosis of lymphocytes [2], and the addition of the polymer Ficoll to the whole blood prevented settling of red blood cells, limiting interference of aggregating red blood cells [4]. However, in addition, such chemicals may induce undesired cellular activation: PHA, for example, is known to stimulate T-cell proliferation [12]. An experimental setup without the addition of cell-preserving chemicals is preferred when studying the natural behavior and effect of blood cells.

A limited number of studies describe the effects of whole blood storage on cellular responses. Unfortunately, these studies only focus on the effects on a specific cell population or cellular response, with contradictory results. The temperature at which the blood is stored may affect the functionality of monocytes, with low temperature storage (4°C for 24 hours) preferred over room temperature [13]. Another study demonstrated that when stored at room temperature, the number of cytokine-producing monocytes remains relatively stable, whereas interferon (IFN)γ- and IL-2-producing T-cells declined during storage [14].

We aimed to provide a comprehensive overview of the effect of sample aging on cell viability and stress and cellular reactivity to exogenous immune triggers. We investigated cell responsiveness by quantification of secreted cytokines, and we looked at the percentage of responsive cells for particular cell subsets (T-cells and monocytes) by means of the flow cytometric detection of intracellular cytokines. To obtain insight into cell-specific or pathway-specific effects of sample aging, we used immune triggers activating different immune cell subsets. T-cells were stimulated by simultaneous incubation with phorbol 12-myristate 13-acetate (PMA) and ionomycin and by staphylococcal enterotoxin B (SEB). PMA plus ionomycin induces a general T-cell activation via protein kinase C (PKC) and nuclear factor of activated T-cell (NFAT) signaling. Superantigen SEB activates up to 20% of all T-cells via the T-cell receptor β chain [15]. Monocyte activation was induced by lipopolysaccharide (LPS), a Toll-like receptor (TLR) 4 ligand.

2. Materials and Methods

2.1. Blood Collection. Blood was collected from healthy volunteers by venipuncture into sodium heparin-coated vacutainers or cell preparation tubes (CPT) containing sodium heparin (Becton Dickinson, NJ, USA) after written informed consent was obtained in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki.

2.2. Whole Blood Incubations. Whole blood was simulated for 3 or 24 hours with LPS (2 ng/mL), SEB (100 ng/mL), or PMA/ionomycin (150 ng/mL and 7.5 μg/mL, resp.). All reagents were obtained from Sigma-Aldrich (Deisenhofen, Germany). For intracellular staining, brefeldin A (Thermo Fisher Scientific) was added to the cultures. Cultures were incubated at 37°C and 5% CO_2.

2.3. PBMC Isolation and Incubations. PBMCs were collected from CPT samples and washed twice with PBS. PBMCs were counted using the MACSQuant 10 analyzer and resuspended at 1×10^6 cells/mL in RPMI1640 supplemented with 10% FBS. PBMCs were stimulated with 2 ng/mL LPS for 24 hours.

2.4. Flow Cytometry. Flow cytometric staining of leukocyte subsets in whole blood cultures was done after red blood cell lysis with RBC lysis buffer (Thermo Fisher Scientific). Leukocytes were incubated with TLR4-APC (clone HTA125, Thermo Fisher Scientific), Annexin V-FITC (Miltenyi Biotec), CD14-VioBlue (clone REA599), and/or CD3-VioGreen (clone REA613) for 30 minutes at 4°C. For intracellular staining, cells are fixed (IC fixation buffer, Thermo Fisher Scientific) and permeabilized (permeabilization buffer, Thermo Fisher Scientific). Then IL-2-PE (clone N7.48 A), TNFα-PE (clone REA656), IFNγ-PE (clone REA600), or IL-6-PE (clone MQ2-13A5) antibodies were added in the presence of Fc Receptor blocking reagent (all from Miltenyi Biotec, Bergisch-Gladbach, Germany, unless indicated otherwise) and incubated at 4°C for 30 minutes. Samples were measured with a MACSQuant 10 analyzer (Miltenyi Biotec). Unstimulated and fluorescence minus one controls for non-lineage markers were used for gate setting.

2.5. Mitochondrial Function. Mitochondrial membrane potential (MMP) was assessed in whole blood cultures after red blood cell lysis with RBC lysis buffer. Leukocytes were incubated with 0.5 μM JC-1 (Mitoprobe kit, Thermo Fisher Scientific) for 15 minutes. CCCP (10 μM) was used as a positive control. MMP was assessed in monocytes and T-cells with a MACSQuant 10 analyzer (Miltenyi Biotec). Mitochondrial function was expressed as the mitochondrial membrane potential, calculated as follows [16]:

$$\Delta\psi m = \frac{(\text{FL1} : \text{FL2})}{(\text{FL1\_CCCP} : \text{FL2\_CCCP})} * 100\%. \quad (1)$$

2.6. Cytokine Measurements. IFNγ, TNFα, IL-1β, and IL-6 from whole blood culture supernatants were measured using the V-plex inflammatory panel-I kit from Meso Scale Discovery (Rockville, MD, USA). IL-2 and IL-10 were measured by ELISA (Thermo Fisher Scientific).

3. Results

3.1. Sample Aging Results in a Rapid Decline of LPS-Driven Responses, Whereas PMA- and SEB-Driven Are Less Affected. Whole blood was collected and stored at room temperature or at 4°C until incubation experiments were started (immediately after blood collection and 0.5, 1, 2, 4, and 10 hours after blood collection). Whole blood cultures were stimulated with LPS, SEB, and PMA/ionomycin. Sample aging strongly reduced LPS-induced IFNγ, IL-1β, IL-6, and TNFα release (Figure 1(a)). A delay in the start of the incubation of only 0.5 hour already resulted in a loss of 30–50% of the cytokine

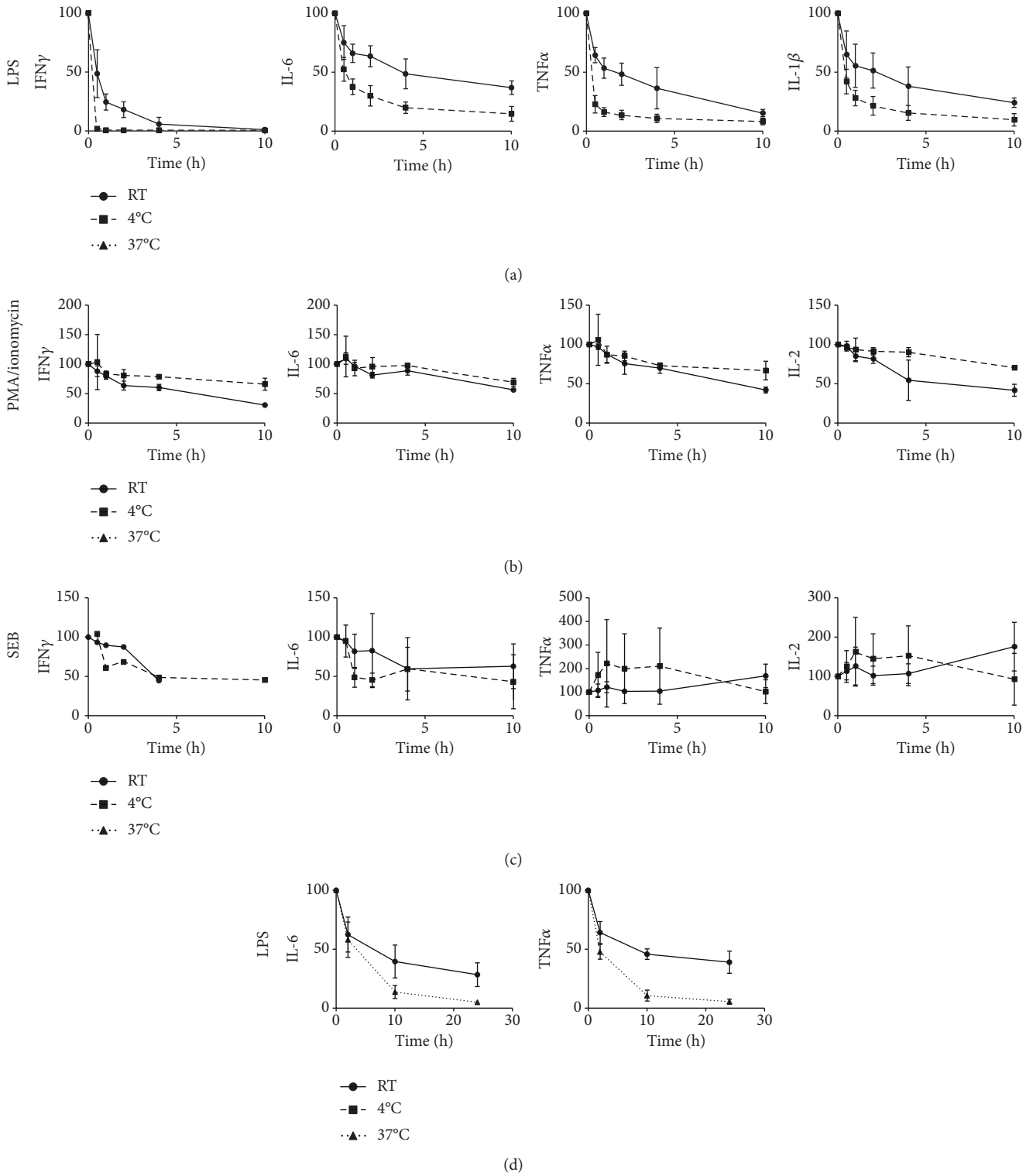

Figure 1: Cytokine release in whole blood culture supernatants (as % of the response at $t = 0$, average plus SD). The whole blood of 3 donors was stimulated for 24 hours with LPS (a), PMA/ionomycin (b), or SEB (c). The whole blood of 5 donors was stimulated for 24 hours with LPS (d). The x-axis indicates the sample age (time span between blood collection and start incubation). The blood was stored at room temperature (continuous line), 4°C (dashed line (a–c)), or 37°C (dashed line (d)).

response when stored at room temperature. When the samples were stored at 4°C, the decrease in cell responses was even more drastic, ranging from 50% for IL-6 to 98% for IFNγ. Interestingly, when the whole blood was stimulated with PMA/ionomycin, again, an effect of sample aging on cytokine release was observed, but this effect was much less

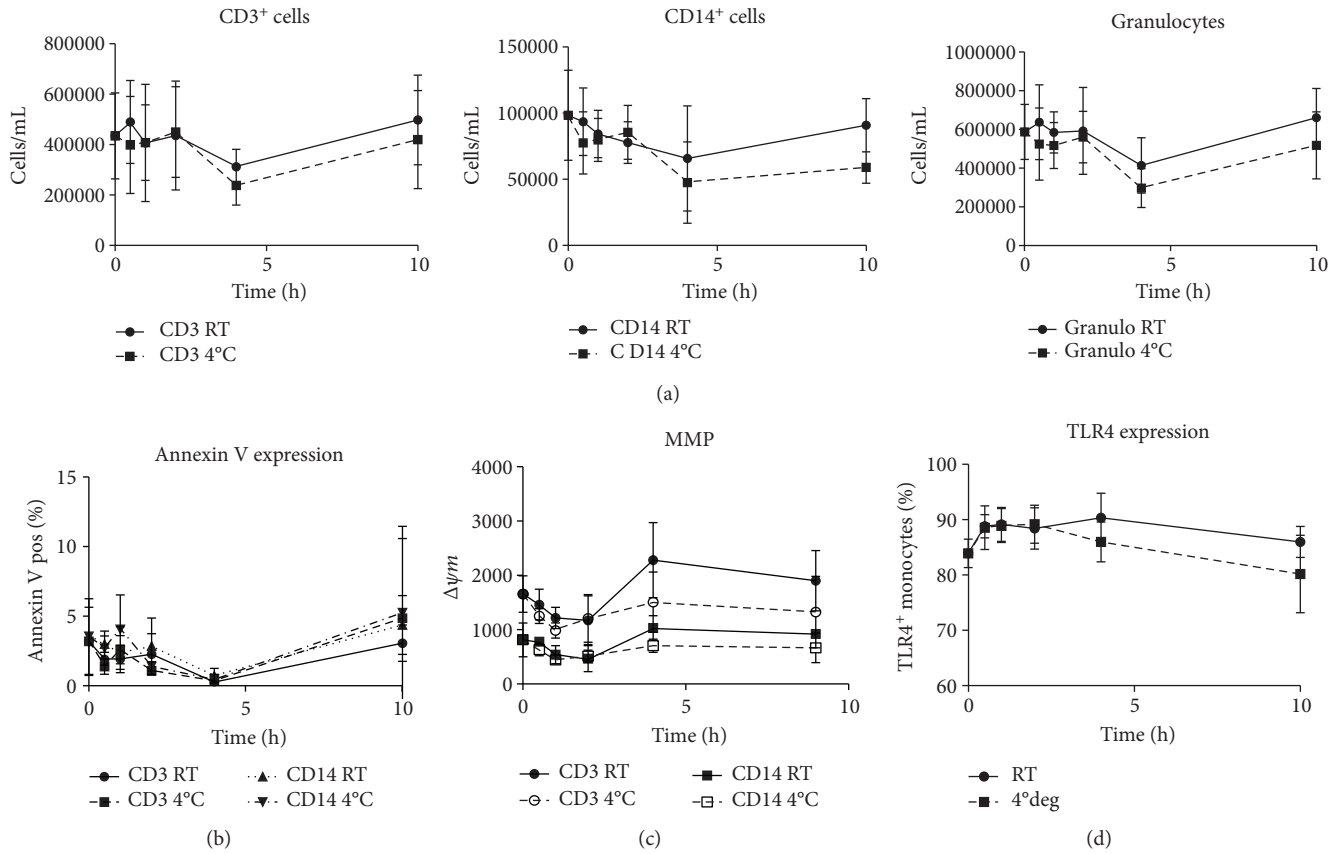

Figure 2: Cell viability (cell counts, annexin V, and MMP) and TLR4 expression in whole blood cultures (average plus SD). In whole blood cultures of 6 donors, absolute cell counts (T-cell, monocyte and granulocyte numbers (a)), apoptosis (annexin V-positive T-cells and monocytes (b)), mitochondrial function (MMP for T-cells and monocytes (c)), and TLR4 expression (TLR4-positive monocytes (d)) were quantified. The *x*-axis indicates the sample age (time span between blood collection and start incubation). The blood was stored at room temperature (continuous lines) or 4°C (dashed lines).

profound (Figure 1(b)). After 0.5 hour, hardly any effect of sample aging was observed. Maximal aging of 10 hours resulted in a loss of 25–60% of the cytokine response (IFNγ, IL-6, and TNFα). In contrast to LPS responses, sample storage at 4°C better preserved PMA/ionomycin-induced cytokine release compared to storage at room temperature. SEB responses were highly variable between subjects, but no strong indications were found for a time-dependent reduction in cell responses (Figure 1(c)). Cytokine release in unstimulated samples remained low (<50 pg/mL) for all sample ages investigated (data not shown). Sample aging did not induce the release of the anti-inflammatory cytokine IL-10 (all responses < 25 pg/mL, data not shown).

Given the large temperature-dependent effect on LPS-driven responses, sample storages at 37°C and at room temperature were compared in a separate experiment (Figure 1(d)). The whole blood was stimulated with LPS immediately after blood collection and after 2, 10, and 24 hours of storage. Sample storage at 37°C resulted in an even more rapid decrease in IL-6 and TNFα production compared to room temperature.

3.2. Sample Aging Does Not Affect Cell Viability, Mitochondrial Function, or TLR4 Expression. Since LPS stimulation results in a mainly monocyte-driven response and SEB and PMA/ionomycin are mainly T-cell stimuli, we hypothesized that monocytes are more prone to undergo cell death due to sample aging. However, absolute counts of $CD3^+$ T-cells, $CD14^+$ monocytes, and granulocytes (as gated in FSC/SSC scatter plot) did not decrease with prolonged sample storage (Figure 2(a)). Also no increase in the number of dead cells was observed, as measured by propidium iodide staining (data not shown). To check whether sample aging rendered the cells apoptotic, annexin V stainings were performed (Figure 2(b)). For all tested conditions, the percentage of annexin V-positive cells remained below 5%, demonstrating that neither monocytes nor T-cells are in a more apoptotic state at the beginning of the culture experiments after prolonged sample aging. Next, the effect of sample aging on cell functionality was investigated by the measurement of the mitochondrial membrane potential (MMP), a marker for cellular stress (Figure 2(c)). Also based on MMP, no effect of sample aging was detected that could explain the significant reductions in LPS-induced cytokine responses. Finally, it was investigated whether the impaired LPS responses in aged blood samples could be explained by a reduced recognition of the trigger. However, sample aging did not significantly affect TLR4 expression on monocytes (Figure 2(d)).

3.3. Sample Aging Does Not Affect the Number of Responding Cells. To explore whether the impaired LPS response after sample aging could be explained by a reduction in responding number of cells, additional experiments were conducted, but now with intracellular cytokine production as the endpoint. Whole blood cultures were started directly after blood collection and with a delay of 1 and 4 hours and storage at room temperature. After LPS stimulation, the percentage of cytokine-producing monocytes was high: approximately 80% produced IL-6, and 95% produced TNFα (Figure 3(a)). T-cells and monocytes remained negative for IFNγ staining after LPS stimulation (data not shown). In response to PMA/ionomycin, approximately 45% of the T-cells produced IL-2 and IFNγ, 30% produced IL-6, and 65% produced TNFα (Figure 3(b)). Monocytes responded to PMA/ionomycin as well: approximately 10% produced IL-6, and 20% produced TNFα. Importantly, sample aging did not affect the number of monocytes or T-cells responding to LPS or PMA/ionomycin (Figures 3(a) and 3(b)).

3.4. The Addition of Culture Medium Does Not Preserve the Whole Blood Response. To assess whether the addition of culture medium prevents the sample aging-dependent loss of cellular responsiveness to LPS, RPMI1640 was added to the whole blood samples directly after blood collection (Figure 4). The addition of RPMI to the blood cultures did not result in the preservation of cell responsiveness: LPS-induced cytokine responses were affected to the same extent by sample aging in the absence and presence of RPMI. To test whether the sample aging effect could relate to influences of dying granulocytes or granulocyte products, an experiment was performed with PBMCs. Cells were stimulated with 2 ng/mL LPS directly after PBMC isolation ($t = 0$ h) or after up to 6 hours of aging (Figure 5). The sample aging effect on LPS responsiveness was comparable between the PBMC experiment (Figure 5) and earlier whole blood experiments (Figures 1 and 4) concerning IL-6 (mild reduction in cytokine release) and IL-1β (significant reduction in cytokine release). Interestingly, the effect on LPS-induced TNFα release was opposite between both settings: whereas sample aging reduced TNFα release in the whole blood (Figures 1 and 4), it strongly enhanced TNFα release in PBMCs (Figure 5).

4. Discussion and Conclusions

Human whole blood cultures are widely used for the investigation of physiological pathways and drug effects *in vitro*. Detailed information on the effect of "sample aging" (the time span between blood collection and experimental start) on the experimental outcome is not readily available in the public domain. This is an important knowledge gap, especially since whole blood-based pharmacodynamic assays have become increasingly important for the guidance of first-in-human clinical pharmacology studies with investigational medicinal products. Whole blood challenges are commonly applied to study the effect of immunomodulatory compounds on innate immune responses, such as ex vivo LPS and PHA stimulations [17–19]. No strict protocols or criteria apply for such whole blood cultures, and the main factor that potentially confounds experimental outcome is the "sample age," the time span between blood collection and the start of the experiment. To provide more insight into the effect of sample aging on innate immune responses induced ex vivo, we stimulated whole blood samples with cell-specific immune triggers (LPS, PMA/ionomycin, and SEB). We studied the effect of sample aging on the ability of immune cells to respond to these triggers. The whole blood was minimally diluted (9% dilution) to resemble *in vivo* conditions as close as possible.

Sample aging at room temperature, but also at 4°C or 37°C, profoundly inhibited LPS-induced cytokine release. At room temperature, the reduction ranged from 20–50% after 30 minutes to 80–100% after 10 hours, with the strongest reductions observed for IFNγ and the smallest reduction for IL-6. An LPS-driven cytokine response in whole blood samples is mainly monocyte-derived [20]. PMA/ionomycin- and SEB-induced cytokine releases, both mainly T-cell-driven, were also reduced by sample aging but to a lesser extent (20–50% after 24 hours). Apparently, in a whole blood setting, monocytes are more affected by sample aging than T-cells. PMA/ionomycin stimulation of the whole blood drives a T-cell response, but also directly activates monocytes. For example, monocytes produce IL-2 upon PMA/ionomycin stimulation [21]. We observed IL-6 and TNFα production by monocytes after PMA/ionomycin stimulation. Therefore, the slight reduction in IL-6 and TNFα in aged whole blood samples stimulated with PMA/ionomycin is probably explained by a lower responsiveness of the monocyte fraction, and not of the T-cell fraction.

Previous reports on the effect of sample aging show that monocyte responses do not diminish after prolonged sample storage [13, 14]. Schultz et al. concluded that prolonged sample storage resulted in a decreased lymphocyte response, without affecting monocyte responsiveness [14]. However, these conclusions were based on intracellular cytokine staining only. In our experiments, intracellular cytokine staining also showed that the number of LPS-responding cells was not impacted by sample aging, so the reduced responsiveness to LPS could not be explained by fewer cells responding to the trigger. In previous experiments, whole blood cultures were diluted with culture medium providing nutrients for the cells [14, 22], which may preserve cell responsiveness during prolonged periods of sample storage. Therefore, we repeated our experiment with RPMI1640 culture medium added to the aging samples, but this did not prevent or reduce sample aging-dependent decreases in cytokine production.

Since monocytes can die via an apoptotic process in the absence of specific activation stimuli [23, 24], we investigated whether reduced LPS responsiveness could be explained by cell death or cellular stress. However, we did not find any indication for a sample aging-dependent reduction in the number of viable immune cells, elevated apoptotic cells, or altered mitochondrial membrane potential. This is in line with literature reporting that apoptosis in whole blood cultures only develops after at least 24 hours of sample storage [3]. Since the uptake of apoptotic cell fragments may suppress the cytokine production by macrophages [25], the conclusion that reduced cytokine responses in our

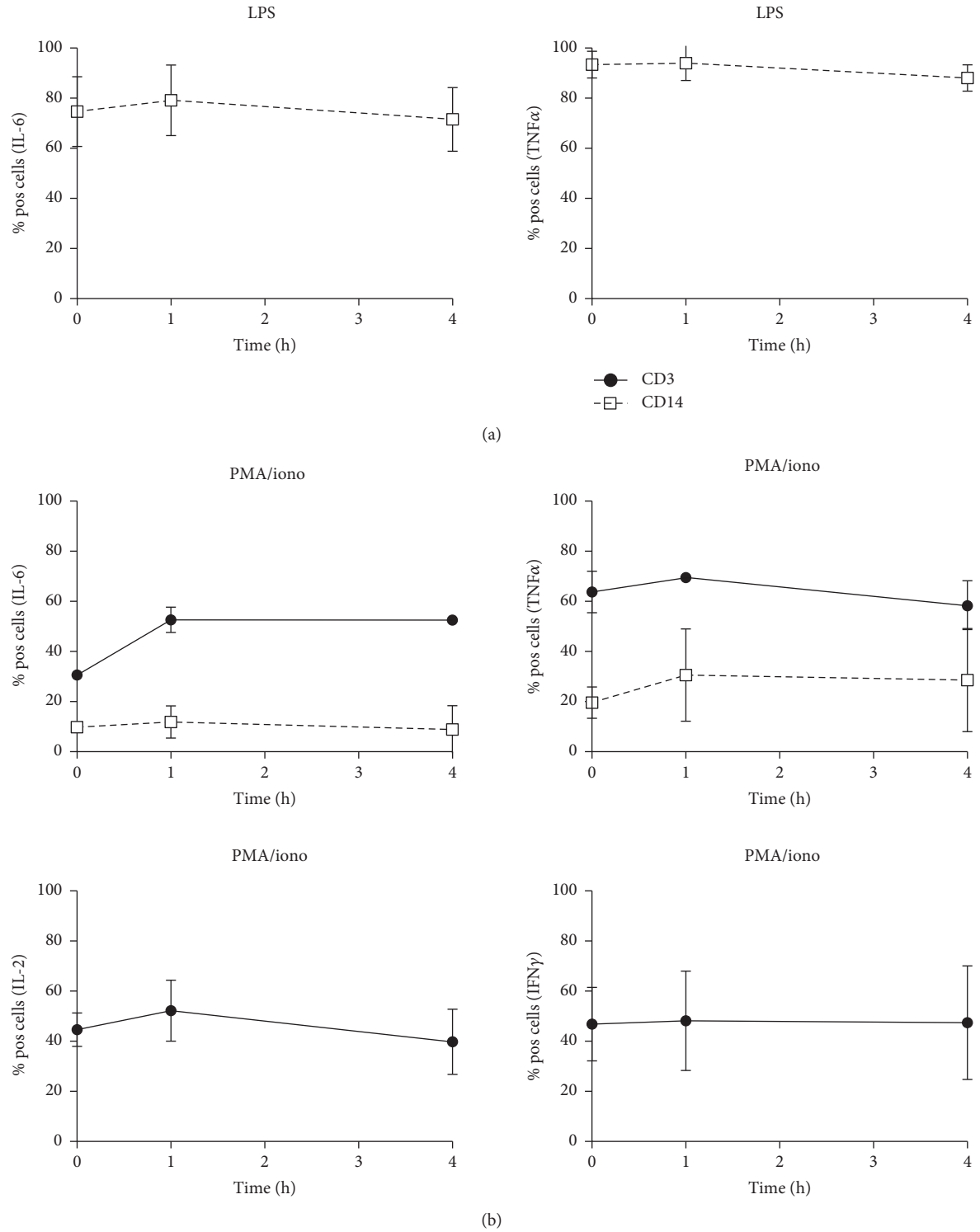

Figure 3: Cytokine-producing cells in whole blood cultures (average plus SD). The whole blood of 5 donors was stimulated for 3 hours with LPS (a) or PMA/ionomycin (b) in the presence of brefeldin A to block cytokine secretion. The percentage of positive cells for intracellular cytokines was quantified for T-cells (continuous lines) and monocytes (dashed lines). The x-axis indicates the sample age (time span between blood collection and start incubation). The blood was stored at room temperature. T-cells and monocytes remained negative for IFNγ staining after LPS stimulation.

experiments do not coincide with cell death or cellular stress which is informative and suggests that other physiological mechanisms may explain the observed effects of sample aging. We also demonstrated that reduced cytokine release in aging samples did not correlate with downregulated TLR4 expression or with an enhanced IL-10 production

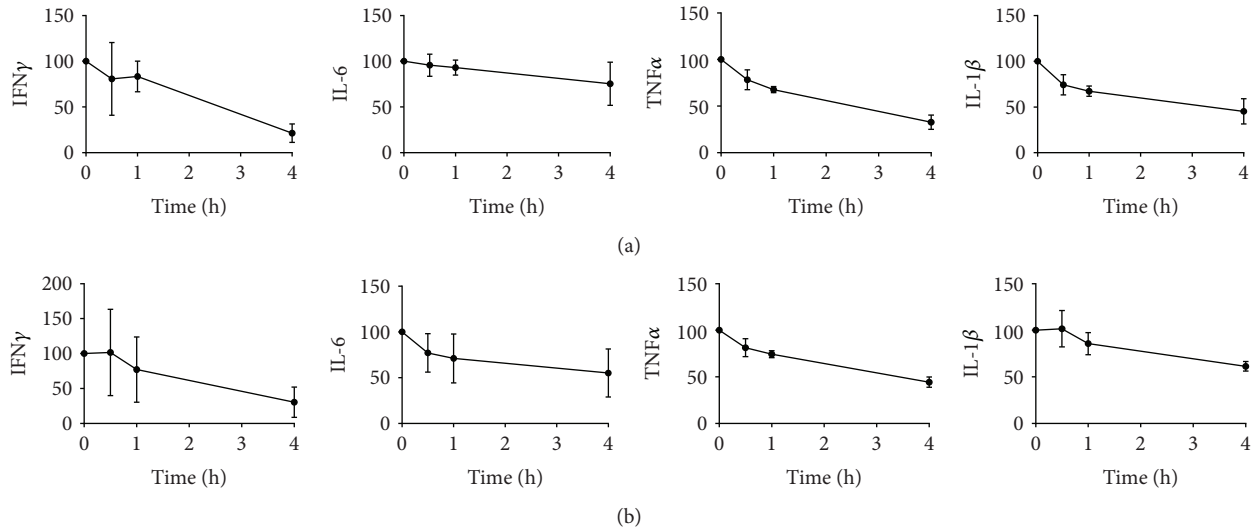

Figure 4: Cytokine release in whole blood culture supernatants; RPMI effect (as % of the response at $t = 0$, average plus SD). The whole blood of 3 donors was stimulated for 24 hours with LPS in the absence of RPMI (a) or diluted 1 : 1 with RPMI during aging (b). The x-axis indicates the sample age (time span between blood collection and start incubation). The blood was stored at room temperature.

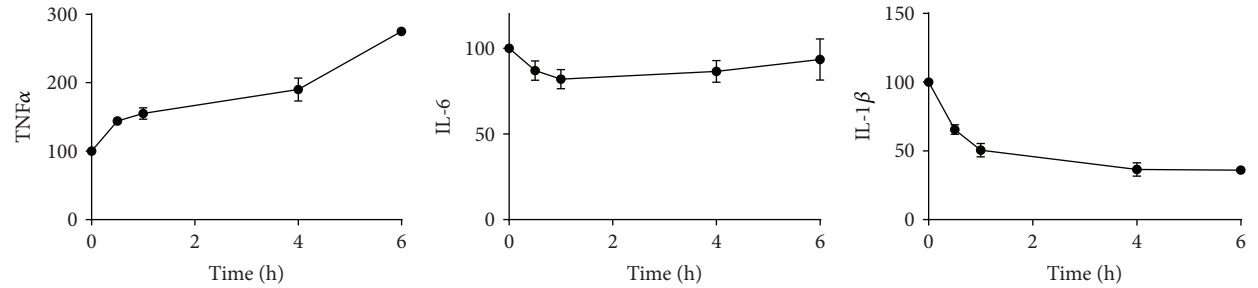

Figure 5: Cytokine release in PBMC culture supernatants (as % of the response at $t = 0$, average plus SD). Isolated PBMCs of 2 donors were stimulated for 24 hours with 2 ng/mL LPS directly after isolation or after aging up to 6 hours in RPMI + 10% FBS. The x-axis indicates the sample age (time span between PBMC isolation and start incubation). Cells were stored at room temperature. For IFNγ, most samples remained below LLOQ.

(data not shown). Thus, we speculate that sample aging induces an inhibitory pathway downstream from TLR4 in monocytes. Alternatively, changes in the expression of cytokine receptors on monocytes may have caused autocrine consumption of cytokines, but this was not investigated.

All the cytokine release experiments described in this manuscript were performed with incubation durations of 24 hours. This time span is sufficiently long to allow not only primary LPS-driven responses but also secondary leukocyte responses initiated by LPS-induced factors or even by factors induced by sample aging. One potentially relevant factor driving secondary responses is granulocyte-related products. For example, granulocyte contamination in PBMC cultures reduced T-cell responses to PHA and fMLP [26]. Granulocytes are known to have a short lifespan and to be sensitive to sample handling. Spontaneous activation of granulocytes occurs after 6–8 hours after venipuncture [26]. In our experiments, no apoptosis of granulocytes was observed in the first 10 hours after sample collection (data not shown). Since we did not assess the level of granulocyte activation, it is theoretically possible that this may have had an effect on the immune responses in our whole blood cultures. However, we showed that sample aging-dependent alterations in some LPS-induced cytokine responses were not only observed in the whole blood but also in PBMC cultures, suggesting that granulocyte-derived factors alone do not explain the observed effects of sample aging.

Significant levels of IFNγ were released upon LPS, SEB, and PMA/ionomycin stimulation in whole blood cultures. However, intracellular staining showed that LPS stimulation did not induce IFNγ production in either monocytes or T-cells. There are different potential explanations for this observation. Possibly, a different cell type accounted for the IFNγ production after LPS stimulation, for example, NK cells and B cells, are known to produce IFNγ upon induction of innate immune responses [27]. Alternatively, LPS-induced IFNγ release may have been a secondary to a primary LPS-driven effect, and the incubation time for the intracellular cytokine experiments was too short to allow such a secondary response. Further investigation into this observation falls beyond the scope of this paper.

These results underline the importance of the use of fresh samples when investigating innate immune responses in the whole blood. Given the ever increasing application of whole

blood challenge tests as pharmacodynamic readout measure in early phase clinical pharmacology trials, a better understanding of the conditions affecting the outcome of such tests is critical. We demonstrated that sample aging primarily affects monocyte responses and that this cannot be explained at the level of cell viability or ligand recognition.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Modulation of Inflammatory Reactions by Low-Dose Ionizing Radiation: Cytokine Release of Murine Endothelial Cells Is Dependent on Culture Conditions

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Background. In many European countries, patients with a variety of chronical inflammatory diseases are treated with low-dose radiotherapy (LD-RT). In contrast to high-dose irradiation given to tumor patients, little is known about radiobiological mechanisms underlying this clinical successful LD-RT application. The objective of this study was to gain a better insight into the modulation of inflammatory reactions after LD-RT on the basis of endothelial cells (EC) as major participants and regulators of inflammation. *Methods*. Three murine EC lines were cultivated under 2D and 3D culture conditions and irradiated with doses from 0.01 Gy to 2 Gy. To simulate an inflammatory situation, cells were activated with TNF-α. After LD-RT, a screening of numerous inflammatory markers was determined by multiplex assay, followed by detailed analyses of four cytokines (KC, MCP-1, RANTES, and G-CSF). Additionally, the monocyte binding to EC was analyzed. *Results*. Cytokine concentrations were dependent on culture condition, IR dose, time point after IR, and EC origin. IR caused nonlinear dose-dependent effects on secretion of the proinflammatory cytokines KC, MCP-1, and RANTES. The monocyte adhesion was significantly enhanced after IR as well as activation. *Conclusions*. The study shows that LD-RT, also using very low radiation doses, has a clear immunomodulatory effect on EC as major participants and regulators of inflammation.

1. Introduction

For the treatment of a variety of chronical inflammatory and painful joint diseases such as heel spurs [1] or osteoarthritis [2] as well as periarthritis humeroscapularis [3], low-dose radiotherapy (LD-RT) is practiced in many European countries [4, 5]. Total doses of LD-RT include 5% to 10% of those given to tumor patients, assuming different radiobiological mechanisms caused by LD-RT compared to high-dose radiotherapy (HD-RT). Whereas HD-RT used in cancer therapy has been proven to induce proinflammatory processes on the immune system [6], after LD-RT, anti-inflammatory and analgesic effects are clinically observed [2, 7, 8]. But the knowledge about radiobiological mechanisms underlying this clinical successful LD-RT application is limited [9]. Therefore, the objective of this study was to gain a better insight into the modulation of inflammatory reactions after LD-RT.

Major participants and regulators of inflammatory processes are known to be endothelial cells (EC) [10]. EC are involved in recruiting of immune cells from the peripheral blood (adhesion) to the inflammation side. Once activated by TNF-α or proinflammatory lipopolysaccharide, EC are responsible for the secretion of many chemokines, cytokines, and growth factors as well as adhesion molecules [11–14]. It was shown that their function is modulated also by irradiation [15]. Previous investigations already showed a reduced adhesion of peripheral blood mononuclear cells (PBMCs) to EC after LD-RT [16, 17]. Several other studies revealed the anti-inflammatory effect of LD-RT on various cells, for example EC, with different responses to the applied radiation doses [18]. In TNF-α-activated EC, a nonlinear expression and activity of compounds of the antioxidative system were described, assuming to contribute to the anti-inflammatory effect [8]. So on the basis of EA.hy926 EC and human umbilical vein endothelial cells (HMVECs), the authors

showed a discontinuous expression and enzymatic activity of glutathione peroxidase accompanied by a lowered expression and DNA-binding activity of the transcription factor nuclear factor E2-related factor 2 (Nrf2) after LD-RT. But most of the *in vitro* studies investigating the effect of LD-RT on EC were performed using two-dimensional (2D) cultivation conditions.

In 2D cultivation, EC grow as a homogenous monolayer on different plastic or glass substrates which do not reflect the physiology of the *in vivo* situation. The morphology of cells as well as cell-cell and cell-matrix interactions are different in tissue or organs compared to flat 2D cell culture conditions [19, 20]. The use of eligible three-dimensional (3D) cell culture models facilitates a tissue or organotypic differentiation of cells. Mechanical and biochemical signals and reactions, communications between cells, or the surrounding matrix are better reestablished under 3D conditions. A deeper insight into migration or adhesion behavior of cells is given likewise in 3D models studying cell physiological reactions to stress-inducing stimuli or effects caused by IR and chemotherapeutic treatment of cells [21]. Experiments have shown differences in the expression patterns of various genes in melanoma cells [22] or human lung fibroblasts [23] as well as mammary epithelial cells [24, 25] when cultured in 3D compared to 2D. Former studies with prostate cancer cells did not only discover different response behaviors of cells; they also demonstrated differences in metabolism and differentiation when cells were cultured in the Matrigel™-based extracellular matrix (ECM) [24–26]. Experiments under 3D culture conditions thus shall give a more detailed picture of inflammatory reactions after exposure to IR, which is more closely reflective to the events *in vivo*. Therefore, for gaining a better insight into the modulation of inflammatory reactions after LD-RT, one main objective in the present study was to investigate the effect of LD-RT on the alteration in cytokine release of EC exposed to single doses of X-rays, with special emphasis on the differences between 2D and 3D culture conditions.

2. Materials and Methods

2.1. Cell Lines and Cultivation. All experiments were performed with three murine EC lines and one murine monocyte cell line. The mlEND1 cells present mesenterial lymph node-derived EC. The H5V cells, derived from the embryonic heart (described in [27]), were kindly provided by Dr. Annunciata Vecchi, Centro Ricerche, Istituto Clinico Humanitas, Rozzano, Italy. The cerebral cortex EC bEND3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The monocyte cell line WEHI-274.1 was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, Cologne, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS, Merck Millipore, Darmstadt, Germany), 100 U/mL penicillin, and 100 μL/mL streptomycin (Sigma-Aldrich, Hamburg, Germany) in 75 cm^2 flasks at 37°C and 5% CO_2.

2.1.1. 2D Cell Cultivation. For the two-dimensional cultivation, cells were seeded in 24-well plates (analysis of pro- and anti-inflammatory cytokines) or 96-well plates (viability assay) and cultured under standard conditions.

2.1.2. 3D Cell Cultivation. For the three-dimensional cultivation, the multiwell plates were precoated with agarose, and cells embedded in growth factor-reduced Matrigel (BD Biosciences, Heidelberg, Germany—now sold by Corning Incorporated, NY, USA) with a 0.5 mg/mL protein concentration were plated on the agarose layer and cultured under standard conditions.

2.2. 2D Monocyte-Binding Assay. EC were seeded in duplicates in 6-well plates for 24 hours and cultured under standard conditions. Subsequently, the medium was replaced with serum-free medium supplemented with or without TNF-α 2 hours before irradiation. After irradiation, cells were kept in the incubator for 24 hours. The nonirradiated WEH1 monocytes were dyed with CFSE (Molecular Probes—now sold by Thermo Fisher Scientific, Schwerte, Germany), for 5 min in the incubator and then were added to EC for 2 hours (static adhesion assay) at 37°C/5% CO_2, followed by several gentle washing steps to remove unbound monocytes. The number of monocytes bound to EC was determined by using the flow cytometer FC500 (Beckman Coulter, Krefeld, Germany).

2.3. TNF-α Stimulation. Prior to radiation, EC were activated with TNF-α (R&D Systems, Wiesbaden, Germany) to stimulate the secretion of inflammatory markers by simulating an inflammation. For that, 24 hours after seeding and 2 hours before IR, the medium was replaced by serum-free medium with or without supplementation of ng/mL TNF-α.

2.4. Ionizing Radiation (IR). At 24 h after seeding, EC were irradiated with X-rays using an Xstrahl 200 therapy system (Xstrahl Ltd., Surrey, United Kingdom; dose rate 0.52 Gy/min and energy 200 kV) at room temperature with single doses of 0.01 Gy, 0.05 Gy, 0.075 Gy, and 0.1 Gy. Cells irradiated with 2 Gy served as positive control. Sham-irradiated samples (0 Gy) were kept at room temperature in the X-ray control room while others were irradiated.

2.5. Analysis of Cellular Metabolic Activity. The metabolic activity of the cells was detected by the water-soluble tetrazolium 1 (WST-1) assay (Roche Deutschland GmbH, Mannheim, Germany) 4 hours, 24 hours, and 48 hours after IR. Cell samples were seeded in triplicates in 96-well plates, TNF-α activated, and grown under standard conditions (see above). After IR, WST-1 reagent was added for 2 hours and the assay's specific instruction was followed for analysis. The tetrazolium salt WST-1 was metabolized into a formazan dye solely by metabolically active cells. The number of vital cells directly correlated with the metabolized formazan dye quantity. The Anthos Zenyth 340r reader (Anthos Mikrosysteme GmbH, Krefeld, Germany) was used for spectrophotometric measurement.

2.6. Analysis of Cytokines

2.6.1. Sample Collection for Cytokine Measurement. For collection of the supernatant, cells were seeded in 24-well plates and cultivated to confluence for 24 hours under standard conditions. 2 hours before IR, the medium was replaced by serum-free medium with or without addition of TNF-α. Cells were treated with radiation using doses ranging from 0.01 Gy to 2 Gy. 30 min and 48 hours after radiation, supernatants were collected and stored at −80°C until measurement of inflammatory cytokines. Samples without cells and only with agarose and Matrigel layer served as a control to assess the system.

2.6.2. Measurement of Inflammatory Cytokines. Cytokine levels of 23 different inflammatory markers (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α) were quantified (data not shown) in supernatants harvested from EC cultivated in 2D as well as 3D models stimulated either with TNF-α or not by using a 23-plex mouse cytokine/chemokine kit from Bio-Rad Laboratories GmbH (Munich, Germany) according to the manufacturer's protocol. After measurement and evaluation, four cytokines with the most significant changes in cytokine expression levels were reexamined in more detail: G-CSF, KC, MCP-1, and RANTES with a customized 4-plex mouse cytokine/chemokine kit (Bio-Rad Laboratories GmbH). Data were acquired using the Bio-Plex® 200 suspension array system and analyzed with the Bio-Plex Manager™ Software (version 4.1).

2.7. Analyses of Relative MCP-1 Expression

2.7.1. RNA Isolation. Total RNA was isolated 4 hours, 24 hours, and 48 hours post-IR from mlEND.1 by using the NucleoSpin® RNA kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. The purity of the isolated RNA was verified using the Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany) with 260/280 and 260/230 ratios as well as real-time PCR with RNA as template to verify the lack of genomic DNA contaminations causing false-positive results during amplification.

2.7.2. Reverse Transcription. Less than 1 μg of total RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas/Thermo Fisher Scientific, Schwerte, Germany) following the manufacturer's instructions.

2.7.3. Quantitative Real-Time PCR. The cDNA was subsequently used in a 20 μl real-time PCR containing TaqMan® Universal PCR Master Mix and TaqMan Gene Expression Assays for monocyte chemoattractant protein-1 (MCP-1) as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Life Technologies, Darmstadt, Germany) as an internal control and 36 ng of cDNA as template. Quantitative real-time PCR was carried out using the 7300 Real-Time PCR System (Applied Biosystems®, Life Technologies, Darmstadt, Germany). All reactions were performed in triplicates. The MCP-1 mRNA expression was normalized to the housekeeping gene GAPDH, which showed no change in expression profile after IR in pretests and therefore served as an internal control. To estimate the relative expression change, all samples were normalized to the control samples at 0 Gy for each time point which were set to 1 using the delta-deltaC_T (2^{-CT}) method (comparative C_T method).

2.8. Statistical Analysis. All data are presented as means ± standard deviation (SD) on the basis of at least three independent experiments. The statistical significance of differences was assessed by the Student t-test. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Influence of LD-RT on Metabolic Activity. The metabolic activity was measured in the three murine EC lines mlEND.1, H5V, and bEND.3 at 4 hours, 24 hours, and 48 hours after IR with different doses. The metabolic activity increased in all tested EC lines over time, but remained unaffected by radiation treatment or activation of the cells with TNF-α prior to IR (Supplement Figure 1). Therewith, all subsequent observed changes in secretion of different markers will be resulted by IR, not by alteration of metabolic activity.

3.2. Effect of LD-RT on Cytokine Secretion. An initial screening of cytokines should ascertain which of the 23 inflammatory markers (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α) were secreted by EC with and without IR and TNF-α. Levels of 23 inflammatory cytokines were quantified in supernatants of three different EC lines. From these 23 measured markers, 18 were below the detection limit of the assay or not detectable at all. Four out of 23 cytokines showed significant changes after IR: keratinocyte-derived chemokine (KC), monocyte chemoattractant protein-1 (MCP-1 or CCL2), regulated on activation, normal T cell expressed and secreted (RANTES or CCL5), and granulocyte-colony stimulating factor (G-CSF).

3.2.1. Effect of LD-RT on Secretion of KC. Secretion of KC from the murine H5V cells was detected from 9.9 pg/mL to 623.4 pg/mL in 2D and 29.4 pg/mL to 439.0 pg/mL in 3D (Figure 1). The most significant changes were observed 30 min after IR in 3D-cultured cells without TNF-α supplementation at doses of 0.01, 0.075, 0.1, and 2 Gy and with TNF-α at a dose of 0.01 Gy. It is clearly to be seen that the irradiation dose of 0.01 Gy caused a significant increase in accumulation of KC in the supernatant of the cultured cells. Activation of the cells prior to IR enhanced the effect. The concentration of the cytokine was higher in 3D-cultured cells compared to the 2D samples. In contrast to these results, the KC values were higher in 2D-cultured cells 48 hours after IR. Without TNF-α only in the 3D samples, a significant reduction of KC was detectable at doses of 0.05 and 0.075 Gy. The addition of TNF-α before IR treatment caused an increase in both 2D and 3D samples with a significant reduced concentration in the 2D samples at 0.075 Gy,

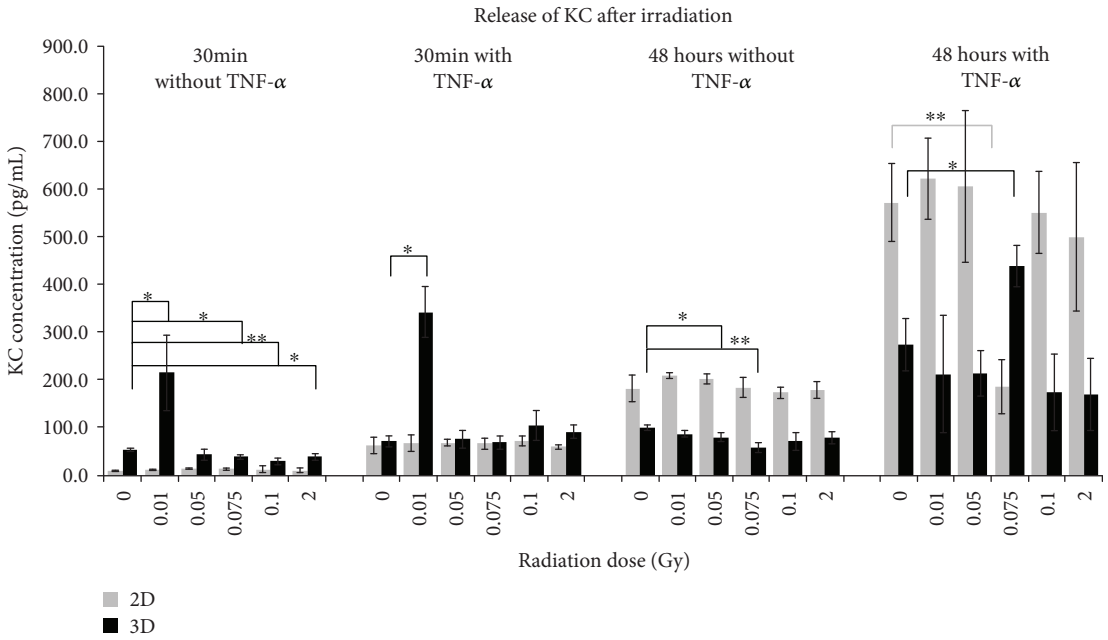

Figure 1: Released levels of the keratinocyte-derived chemokine (KC) in supernatant of H5V endothelial cells. The cytokine concentration was determined by multiplex assay at two time points after irradiation with low doses of X-rays. Changes in cytokine concentrations are presented as mean (pg/mL) ± standard deviation (SD) from three independent experiments; asterisks illustrate significance: $^{*}p < 0.05$ and $^{**}p < 0.01$.

but a significant increase in the 3D samples at the same irradiation dose.

In mlEND.1 cells, the KC concentration in each experimental approach was similar, independently from time point, culture condition, or TNF-α (3D) and time-dependently increasing under 2D conditions (Supplement Figure 2A). In the bEND.3 cells, an increase from 30 min after IR without TNF-α to 48 hours after IR was observed under both culture conditions without significant changes (Supplement Figure 2B).

3.2.2. Effect of LD-RT on Secretion of MCP-1. Secretion of MCP-1 from the murine bEND.3 cells was detected from 37.5 pg/mL to 10,898.2 pg/mL in 2D and from 854.6 pg/mL to 16,212.3 pg/mL in 3D (Figure 2). It is clearly to be seen that the accumulation of MCP-1 was highest under all experimental approaches when cells were cultured in 3D compared to the samples from 2D. The addition of TNF-α before IR caused no significant increase in concentration 30 min after irradiation, but 48 hours after, the MCP-1 concentration reached a maximum in the 3D samples, but without significant change. Compared to these results, cells from the 2D condition secreted MCP-1 significantly higher 30 min after IR at doses of 0.075 Gy and 2 Gy compared to 0 Gy, when TNF-α was supplemented. In contrast to this result, the MCP-1 secretion was significantly reduced 48 hours after IR at all tested doses compared to the sham-irradiated samples under the same culture conditions.

In H5V cells, a time- and TNF-α-dependent increase in MCP-1 was observed, but with no significant changes in 2D. Under 3D conditions, the effect was less distinctive, but with very significant changes 30 min after IR at a dose of 0.01 Gy. TNF-α supplementation enhanced this effect (Supplement Figure 3A). The mlEND.1 cells secreted MCP-1 in a time-dependent manner under 2D as well as 3D conditions, but only 30 min after IR, a significant change was visible (Supplement Figure 3B).

3.2.3. Effect of LD-RT on Secretion of RANTES. Secretion of RANTES1 from the murine mlEND.1 cells was detected in a range from 2.6 pg/mL to 1515.2 pg/mL in 2D and from 18.3 pg/mL to 53.6 pg/mL in 3D (Figure 3). RANTES was secreted by these cells under 2D conditions in a time-dependent manner with a maximum of concentration 48 hours after IR and additional TNF-α. Significant reduction was observed at this time point, but without TNF-α compared to the 0 Gy sample. When cells were cultured in a 3D system, the RANTES accumulation in the supernatant was not as high as in the 2D samples; a clear time-dependent increase was also not observable. TNF-α supplementation only caused a significant higher secretion 30 min after IR at a dose of 0.01 Gy whereas without, significantly reduced concentrations were measured 30 min after IR with doses of 0.075 Gy and 48 hours after IR at doses of 0.01, 0.05, and 0.1 Gy.

In H5V cells, RANTES was secreted time-dependently; TNF-α did not enhance the effect significantly (2D). When cells were cultured in a 3D environment, the RANTES concentrations showed the same level as in 2D, without a significant time- or dose-dependent tendency (Supplement Figure 4A). In bEND.3 cells, the same effect was observed under 2D conditions, with a slightly higher concentration

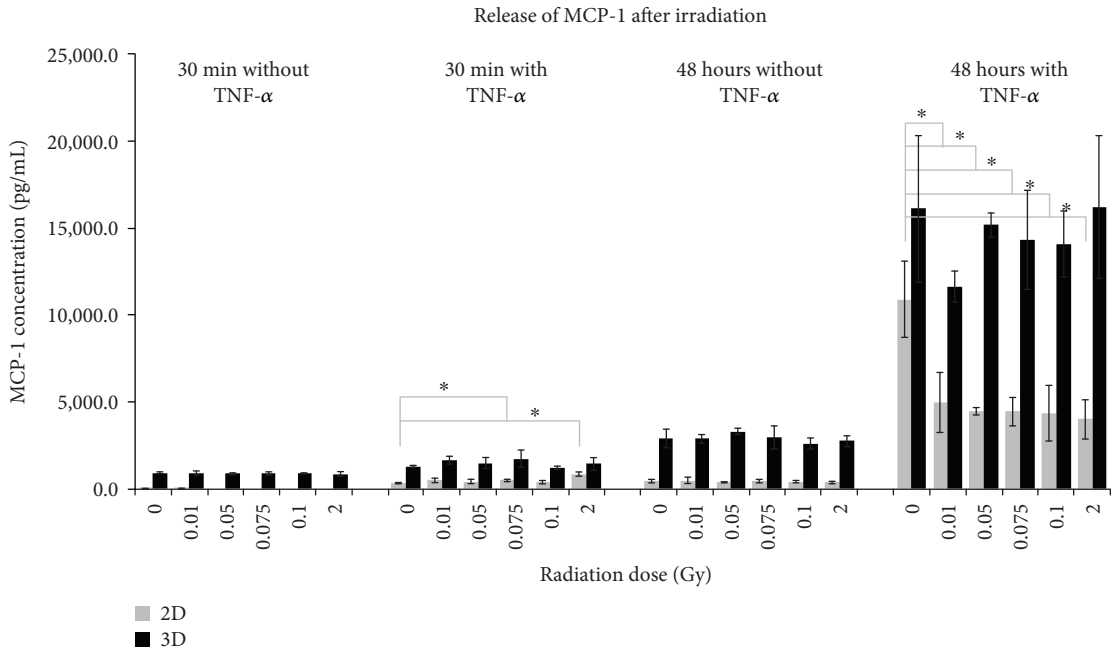

Figure 2: Released levels of monocyte chemoattractant protein-1 (MCP-1) in supernatant of bEND.3 endothelial cells. The cytokine concentration was determined by multiplex assay at two time points after irradiation with low doses of X-rays. Changes in cytokine concentrations are presented as mean (pg/mL) ± standard deviation (SD) from three independent experiments; asterisks illustrate significance: $^{*}p < 0.05$.

compared to H5V cells. Under a 3D culture environment, RANTES was secreted at a similar level without significant time- or dose-dependent changes (Supplement Figure 4B).

3.2.4. Effect of LD-RT on Secretion of G-CSF. If cultivated with a conventional 2D system, only mlEND.1 cells 48 hours after IR and TNF-α treatment alone secreted G-CSF (Supplement Figure 5A). In the 3D model, however, G-CSF concentrations were measurable in the supernatants of mlEND.1 and bEND.3 cells (Supplement Figure 5A) at both time points after IR (mlEND.1: 6.67 pg/mL to 39 pg/mL; bEND.3: 4.41 pg/mL to 9.43 pg/mL), but the difference between nonactivated and activated samples was less distinctive 30 min after IR compared to 48 hours later and only with mlEND.1 cells was the level of G-CSF increased 48 hours after IR. Both cell lines secreted the inflammatory marker in a nonlinear dose-dependent manner. Significant changes were measured 48 hours after IR. In the supernatant of H5V cells, G-CSF was not detectable.

3.3. Effect of LD-RT on Monocyte Binding. Sham-irradiated, nonactivated samples served as a control and were set to 100%. Monocytes remained nonactivated and nonirradiated. Under 2D cultivation conditions, the adhesion of the monocytes to nonactivated mlEND.1 cells resulted in a maximum level at 2 Gy, but with no significant influence of IR (Figure 4(a)). In nonactivated H5V cells, a significant higher adhesion after IR was detected at 0.1 Gy and 2 Gy compared to 0 Gy control. No significant differences in WEH.1 adhesion after IR were observed for nonactivated bEND.3 cells. The supplementation with TNF-α prior to IR resulted in an enhanced adhesion in all three cell lines (Figure 4(b)). With mlEND.1 cells, the adhesion was in average 200% higher compared to the nonactivated, sham-irradiated sample, but a significant influence of LD-RT was not detected. The adhesion of WEH.1 cells to H5V cells after IR was significantly higher at 0.025 Gy, 0.1 Gy, and 2 Gy as well as at 0.075 Gy compared to sham-irradiated, but activated samples. With bEND.3 cells, the adhesion after IR was significantly enhanced after doses of 0.025 Gy, 0.1 Gy, and 2 Gy. The 3D model used in this study for all other experiments—measurement of metabolic activity, cytokine secretion, as well as expression of MCP-1—was not applicable for investigation of monocyte binding to EC under 3D conditions after LD-RT.

3.4. Effect of LD-RT on Relative MCP-1 Expression. To examine if the exposure of EC to LD-RT can not only alter the protein secretion but also influence the expression of inflammatory markers on an RNA level, a qRT-PCR for the MCP-1 gene was performed. Under 2D culture conditions, as shown in Figure 5(a), a significant upregulation of MCP-1 mRNA could be detected 4 hours and 24 hours after IR. A statistically significant expression change was not observed 48 hours postirradiation. The expression pattern of MCP-1 mRNA under 3D culture conditions differed from the pattern in the 2D model (Figure 5(b)). Without activation of the cells, a significant upregulation was detected 24 hours after IR at a dose of 0.075 Gy. In activated cells, the MCP-1 mRNA expression was significantly downregulated 24 hours after IR at a dose of 0.05 Gy and significantly upregulated again 48 hours after IR at a dose of 0.075 Gy.

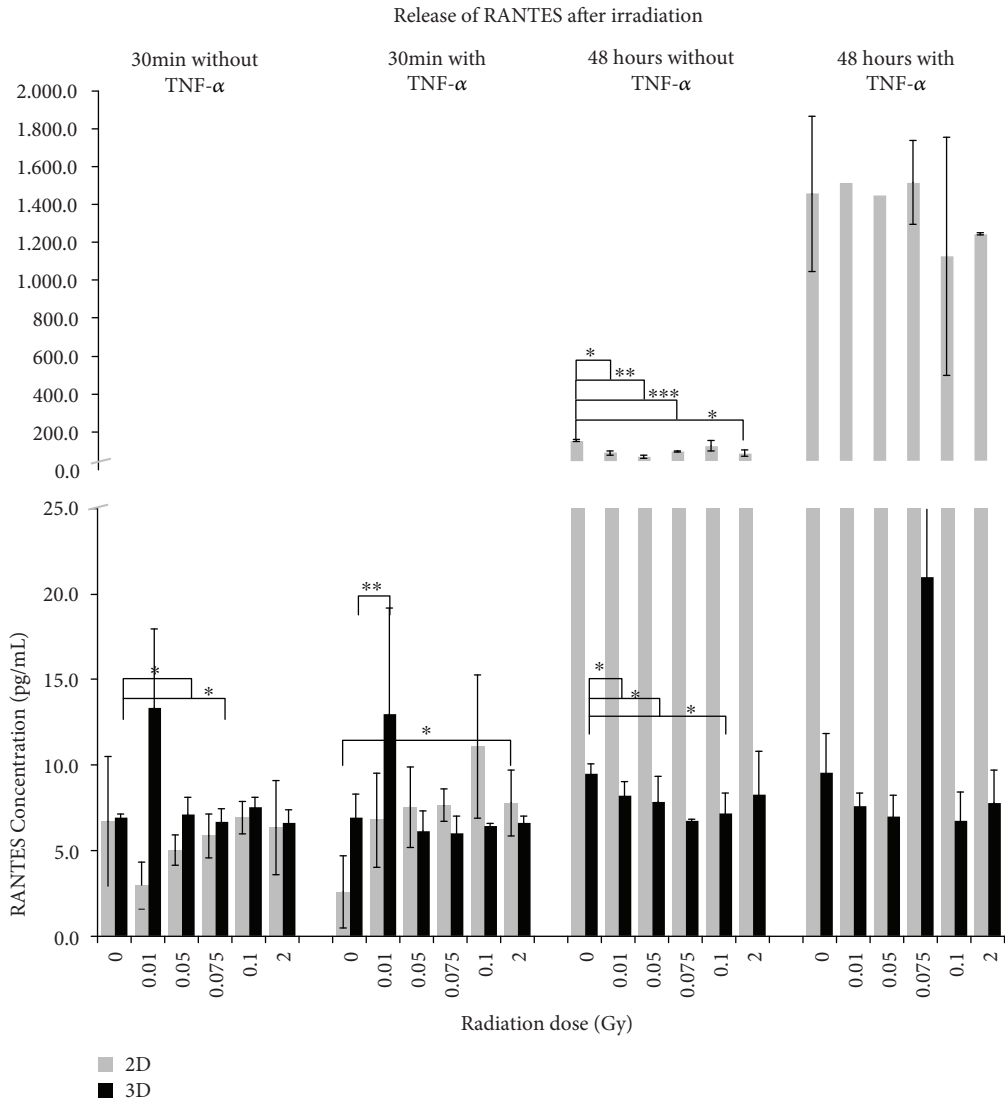

Figure 3: Released levels of RANTES in supernatant of mlEND.1 endothelial cells. The cytokine concentration was determined by multiplex assay at two time points after irradiation with low doses of X-rays. Changes in cytokine concentrations are presented as mean (pg/mL) ± standard deviation (SD) from three independent experiments; asterisks illustrate significance: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

4. Discussion

Cytokines and chemokines are known to contribute to all aspects of inflammatory reactions in different ways and interact with EC and other cells of the immune system [28–33]. The crosstalk between the molecules is of complex nature, and they can have an additive, synergistic, or antagonistic effect on the same process [34]. Among the 23 analyzed inflammatory markers in our study, four (KC, MCP-1, RANTES, and G-CSF) were found to be clearly altered. The results could demonstrate that the secretion of the different cytokines is mainly dependent on (I) the culture condition of the cells, (II) the origin of the cells, (III) the radiation dose, and (IV) the activation with TNF-α.

An alteration of metabolic activity as a reason of the observed changes in secretion of different markers could be excluded because of results from analyses of metabolic activity. We could demonstrate that the cell irradiation up to single doses of 2 Gy and until 48 hours after IR did not affect the metabolic activity significantly. Also, no significant changes were observed after activation with TNF-α. The results verified the preserved metabolic activity of all three EC after IR. The increase in metabolic activity was solely explained by normal cell growth over the observed time frame and therefore the gain of viable cells metabolizing the chemical. The cell division rate of the mlEND.1 cells is higher than the ones from bEND.3 and H5V. This method was used by many other authors like Cervelli et al. whose team irradiated human umbilical vein endothelial cells (HUVEC) with low-energy X-rays in single as well as fractionated low doses and also observed no significant loss of cell viability caused by IR [35].

One main goal of this study was the comparison of cytokine secretion from EC cultivated under 2D or 3D conditions

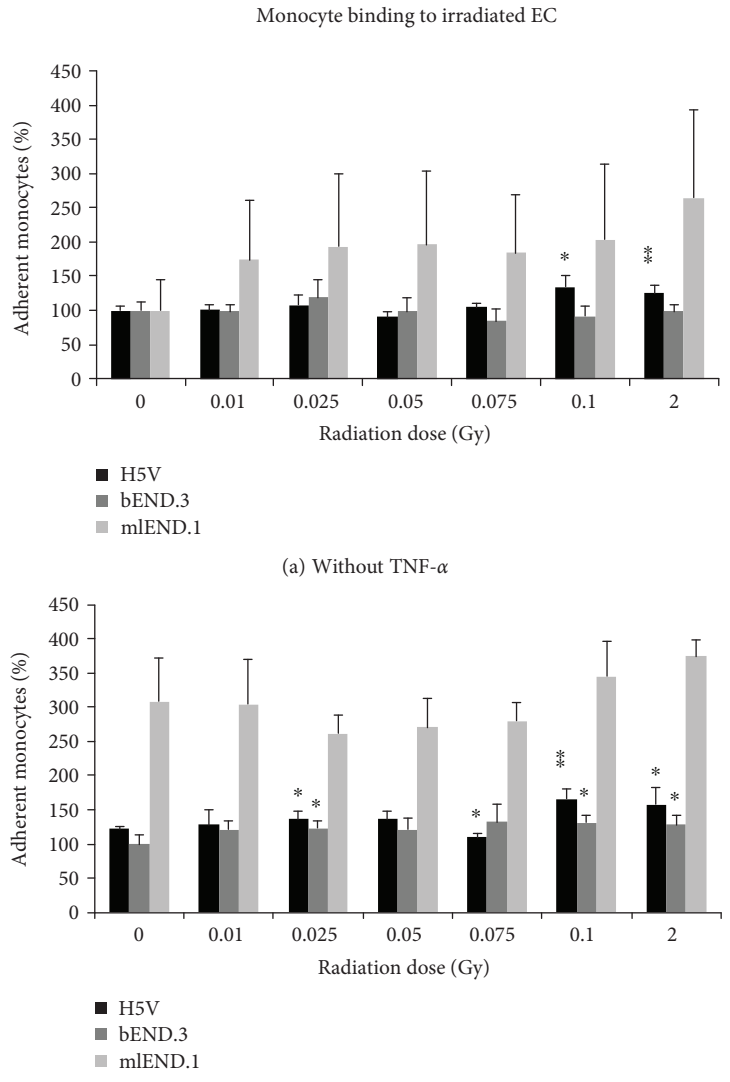

Figure 4: Adhesion of WEH.1 monocytes to irradiated EC (a) nonactivated and (b) activated with TNF-α prior to IR. The percentage adhesion of monocytes to EC was determined by flow cytometric analysis 24 hours after irradiation with low doses of X-rays and normalized to the sham-irradiated, nonactivated samples. Changes in monocyte binding are presented as mean ± standard deviation (SD) from four independent experiments performed in duplicates; asterisks illustrate significance: $^{*}p < 0.05$ and $^{**}p < 0.01$.

and to investigate which culture system is more suitable to study the effect of LD-RT on EC. It was observed that altered releases of inflammatory cytokines were detectable in both culture models, 2D and 3D, but with clear differences. The proinflammatory marker G-CSF was clearly evident in the 3D model in mlEND.1 and bEND.3 cells. But under 2D conditions, G-CSF was measured only in mlEND.1 cells at very low concentrations. G-CSF plays a major role as regulator of haematopoiesis and innate immune responses and is well known to also promote angiogenesis and improves cardiac function [36, 37].

A key chemokine, which plays a pivotal role in the innate immunity, the pathogenesis of various infectious, and inflammatory diseases by attracting monocytes to the site of inflammation or injury, is the monocyte chemoattractant protein-1 (MCP-1) [38]. MCP-1 can be secreted by EC in response to signals like proinflammatory stimuli or IR. Therefore, this molecule is of high interest for low-dose IR research and how it contributes to the modulation of inflammatory reactions especially after LD-RT. In our study, the secretion of MCP-1 was also higher in 3D-cultured cells compared to the samples collected under 2D conditions. Using 2D conditions, the maximum concentration was measured in mlEND.1 and H5V cells in average, but highest in bEND.3 cells when cultured in a 3D model. The murine chemokine KC, a potent chemoattractant for neutrophils and involved in murine inflammatory processes [39], was secreted by mlEND.1 cells highest under 3D conditions, whereas in H5V and bEND.3 cells, 2D-cultured cells secreted this marker in highest concentrations. RANTES is a chemokine with inflammatory properties in a various number of tissues [40] and acts as a mediator of acute and chronic

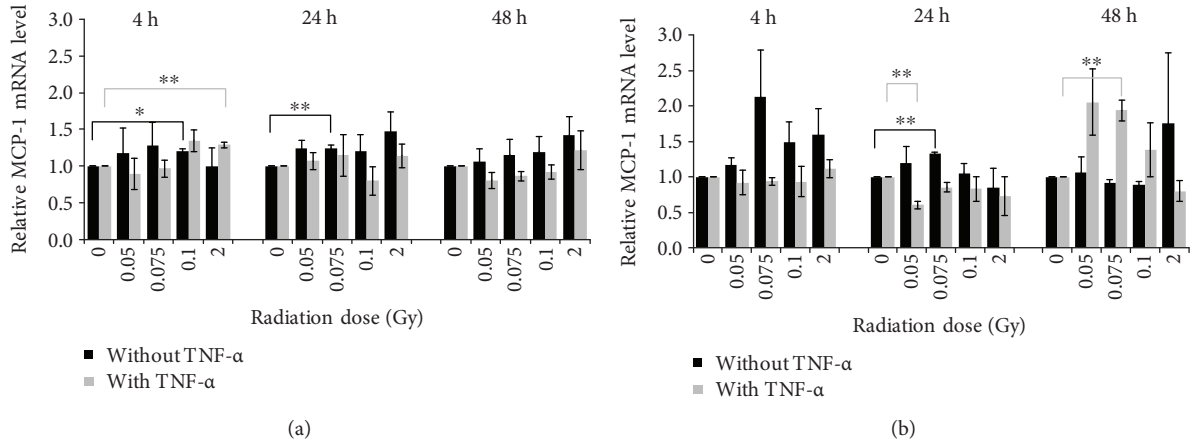

Figure 5: Relative MCP-1 mRNA expression level of (a) 2D- and (b) 3D-cultured mlEND.1 cells. The cytokine expression was determined by quantitative real-time PCR at three time points after irradiation with low doses of X-rays. Changes in cytokine expression are presented as mean (pg/mL) ± standard deviation (SD) from four independent experiments. The expression of MCP-1 was normalized to the housekeeping gene GAPDH; asterisks illustrate significance: $^{*}p < 0.05$ and $^{**}p < 0.01$.

inflammation [29, 38]. All 3 cell lines secreted RANTES in the highest level in a 2D environment. In a study without IR, published by Ghosh et al., the expression of several genes in NA-8 cells (metastatic melanoma cells) was examined under 2D and 3D conditions, and a clear difference between both culture systems was also observed [22]. As an example, the expression of CXCL1 and IL-8 was significantly upregulated in the 3D model when compared to the 2D culture samples, as well as the secretion of the corresponding proteins. The expression of basic FGF, however, was downregulated. Cells in the organism are surrounded by tissue containing a variety of other cells and ECM. This allows the cells to communicate with neighboring cells as well as interact with the components of the ECM. In a conventional 2D experimental set-up, the tissue-specific architecture and biochemical and mechanical cues are lost and do not represent the correct *in vivo* conditions [41, 42]. To reestablish physiological interactions and a similar *in vivo*-like environment for the cells, many different 3D culture models were developed in recent years, including for investigation of radiation effects [19, 21]. Results received in this study were adapted from a Matrigel-based ECM cell culture model and compared to a conventional flat 2D cell culture model. Clear differences in the secretion of cytokines could be observed, not only depending on the culture environment but also depending on the origin of the cells.

For the present study, EC from different origins were used: embryonic heart (H5V—microvascular), mesenterial lymph node (mlEND.1—macrovascular), and frontal cortex (bEND.3—microvascular). The results showed that the secretory potential of EC after IR is dependent on the origin of the cells. In general, it could be observed that cells derived from large vessel (mlEND.1) responded with increased secretion of proinflammatory markers. Cells derived from the microvasculature (H5V) were affected less distinctive by LD-RT, secreted proinflammatory markers in lower concentrations compared to other EC, or did not secrete them at all.

It is well known that the adhesion of leukocytes to the endothelium is one of the initial events during tissue invasion and therefore contributes to inflammatory reactions. This event is mediated by various molecules [43, 44]. Several studies already revealed the reduced adhesion of peripheral blood mononuclear cells (PBMCs) to EC after LD-RT [16, 17]. In the study by Rödel et al., the adhesion of PBMCs to activated EA.hy926 EC cells was significantly reduced 24 hours after IR with 0.5 Gy [16].

Kern et al. published a study in 2000, where they examined if the adhesion capacity of murine EC (mlEND.1) is influenced by their activation status during IR [17]. The adhesion of PBMCs decreased by about 20–30% 24 hours after IR with 0.1 Gy and 0.5 Gy. In our study, we could show that the adhesion of monocytes to nonactivated EC not only was influenced by LD-RT alone but also was enhanced when cells were activated before IR. In contrast to the experimental set-up used in the studies mentioned above, we used X-rays in a very low-dose range of 0.01 Gy to 0.1 Gy as well as 2 Gy as the highest dose.

The 3D model used in this study for measurement of metabolic activity, analyses of cytokine secretion, as well as expression of MCP-1 was not transferable to investigate the monocyte binding to EC after LD-RT. The system used in this study was based on Matrigel with a concentration of 0.5 mg/mL protein. In the experimental set-up, it was not distinguishable if the monocytes were completely migrated through the ECM layer to bind to the EC or if the cells were sticking to components of the layer. Therefore, the results show that a cell culture system based on a simple Matrigel layer is not suitable for all kinds of experimental setups, for example, to investigate the adhesion of monocytes to EC.

We could also demonstrate significant changes in the secretion of the inflammatory markers depending on LD-RT with a specified IR dose. All three EC lines tested responded to various low doses of IR with a nonlinear dose-dependent secretion. These findings could be observed in both culture models. It is well known that high doses of IR result in proinflammatory reactions whereas low doses can cause both, anti-inflammatory and proinflammatory reactions. The effects of high- and low-dose IR on the immune system

and the controversial results obtained with LD-RT not only in *in vitro* but also in *in vivo* experiments were summarized on the model of dendritic and T cell interaction [39].

Over the past decades, many other studies revealed the anti-inflammatory effect of LD-RT on various cells with different responses to the applied radiation doses, which were summarized by Rödel et al. [18].

The four markers examined are commonly known to be involved in inflammatory reactions caused by stimuli like IR [12] as well as TNF-α [45, 46]. It is already known that TNF-α has an effect on the vascular endothelium and on leukocyte interactions [47]. This proinflammatory cytokine may also be involved in the development of cardiovascular diseases, for example, atherosclerosis or congestive heart failure [48]. As suggested, the activation resulted in a distinctly higher release of inflammatory markers by all EC in both 2D and 3D cell culture systems compared to inactivated cells in our study. In a publication by Gerhardt et al. [48], an increase in proinflammatory markers like RANTES or MCP-1 by HUVECs after stimulation with a higher concentration of TNF-α (20 ng/mL) was observed. We could show this effect already with a 50% lower concentration (10 ng/mL). Another difference between the studies is the usage of a 3D-based cell culture model, in which we could demonstrate a much higher secretion of MCP-1 by EC compared to results obtained in the 2D model.

Additionally, to investigate a possible time- and dose-dependent change of cytokine expression on an mRNA level, qRT-PCR was performed with samples from mlEND.1 cells cultured under 2D as well as 3D conditions. It has already been revealed that various cells, cultured in both systems, respond to stimuli with an altered gene expression. IMR-90 cells (human fetal lung fibroblast cells) express genes like IL-8, CXCL1, CXCL2, or VEGF in higher levels under 3D culture conditions compared to samples from the 2D model whereas thrombospondin 1 (THBS1) was expressed at higher levels when cells were examined using the 2D model [49]. The metastatic melanoma cell line NA-8 also expressed various markers in different levels when cultured under 2D or 3D conditions. The gene expression of CXCL1, IL-8, and MIP-3 α was higher in 3D compared to samples of the 2D culture system also [22]. The expression pattern of MCP-1 in mlEND.1 cells was found to be noticeably different than protein secretion at the same time points and applied doses. Our findings suggest that MCP-1 underlies different post-transcriptional and posttranslational mechanisms, which could explain the diverging results obtained on a protein and mRNA level. In 2010, Shebl et al. analyzed various mRNA-protein correlations in PBMCs by testing 22 various cytokines. They illustrated a wide range of correlation between proteins secreted by the cells and the expression of the gene varying from marker to marker and also assumed the regulatory mechanisms after transcription and translation [50]. The transcription rates, splicing mechanisms, protein processing or degradation, or message turnover are just a few of many regulation mechanisms that can influence cytokine gene expression. Stimuli for inducing the expression are distinct and dependent on the cytokine. Possible mechanisms were summarized by Keene in 2007 where ribonucleoproteins (RNP) play a key role in mRNA processing, from transcription to protein synthesis [51].

A possible explanation for the different results detected in 2D and 3D might be the influence of the surrounding matrix, in which the cells are embedded. The ECM pore architecture and rigidity might affect the efficiency of growth factors and other molecules, like TNF-α, to diffuse through the environment. This influences the concentration gradient as well as the protein matrix binding. In the 2D model, cells are exposed to TNF-α almost completely, whereas cells in the 3D model are exposed to an attenuated concentration of the proinflammatory stimuli. This might affect the release and expression of inflammatory markers. Even the composition of the ECM will have an influence on not only the cell morphology and differentiation, but it may also affect the secretion of inflammatory markers. The manufacturer relinquished a vague content for the ECM used in this study which contains entactin, collagen IV, laminin, and heparin sulfate proteoglycan in different concentrations. It is well known that cytokines can interact or bind to components of the ECM, for example, proteoglycans [52]. Previously, the interaction of TNF-α with the ECM glycoproteins laminin (LN) and fibronectin (FN) was described, including how the interactions affected the activity of the proinflammatory protein [53]. Therefore, TNF-α was bound to both ECM components and possibly strengthened the binding of, for example, lymphocytes, to the ECM and promoting cell activation.

In the present paper, we focused on the radiation-induced effect of very low radiation doses because of clinical efforts to further dose reduction in LD-RT to minimize stochastic effects and possible carcinogenic late risks of LD-RT. For decades, LD-RT has been successfully applied using fraction doses of 0.5–1.0 Gy (total doses of 3–6 Gy). However, an optimal RT regimen is still not clear and under current discussion [54]. Additionally, due to discussion about a possible carcinogenic late risk, the application of LD-RT is still a subject of controversial debate and less accepted in many countries [55, 56]. For these reasons, a reduction in radiation doses in LD-RT is an impact of many efforts both clinically and experimentally.

In the last years, there were performed several clinical trials to reduce radiation doses in the treatment of inflammatory and painful joint diseases [1, 54, 57, 58]. According to these results, a new standard for the radiotherapeutical treatment of, for example, painful heel spur, was established with lowered fraction doses.

Recent laboratory studies have shown that single doses <0.1 Gy might be also effective for the treatment of benign diseases. It could be demonstrated that in vivo LD-RT has an impact on the functional as well as quantitative parameters of murine splenocytes [59]. A moderate decrease in the apoptosis of murine dendritic cells after whole body irradiation with low doses of 0.01–0.1 Gy was found. These observations were likewise associated with alterations of the cytokine milieu, including partial downregulation of IL-4 and IFN-γ. Liu et al. showed a stimulated expression of CD80 and CD86 on murine APCs after whole body irradiation with 0.075 Gy and increased IL-12 secretion

4 hours after IR [60]. Additionally, they were able to demonstrate that the expression of CD28 on T cells was upregulated and that of CTLA-4 was downregulated in early time points, also after very low radiation doses (0.075 Gy). In our study, we also could show that immune modulatory effects can be observed after irradiation with very low doses too. These findings may contribute to support of clinical efforts for further dose reduction to minimize stochastic effects and possible carcinogenic late risks of LD-RT.

5. Conclusions

Inflammation is a complex mechanism, and two important functions are the regulation of leukocyte migration and activation by cytokines. EC play a key role in these processes. After activation with appropriate stimuli, they can produce different adhesion molecules and inflammatory mediators like cytokines and chemokines. Little is known about the immune modulatory effects after LD-RT and the effects of these on EC function.

The results obtained in this study indicate an immune modifying ability of LD-RT regarding the response of EC not only in a conventional 2D culture system but also in an ECM-based 3D model. It has been clearly observed that the change of inflammatory cytokine release and monocyte binding to ECs was reliant on the origin of the EC, the radiation dose applied, and the time point after irradiation as well as TNF-α stimulation. For the proinflammatory cytokines KC, MCP-1, and RANTES, a dose-dependent concentration could be observed. LD-RT and/or TNF-α activation resulted in both reduction and increase of the cytokine levels. The monocyte adhesion was significantly enhanced after IR as well as activation. In the present study, the altered release of inflammatory cytokines was detectable in both culture models, 2D and 3D, but with clear differences.

With the results of the present study on the basis of EC, a better insight into the modulation of inflammatory reactions after LD-RT could be given. It was shown that 3D cultivation conditions are not only suitable but also advantageous for the investigation of modulation of inflammatory reactions after LD-RT and can be used for further studies.

Abbreviations

2D: Two-dimensional
3D: Three-dimensional
BBB: Blood-brain barrier
bFGF: Basic fibroblast growth factor
cDNA: Complementary deoxyribonucleic acid
CFSE: Carboxyfluorescein succinimidyl ester
DMEM: Dulbecco's modified Eagle's medium
EC: Endothelial cells
ECM: Extracellular matrix
FCS: Fetal calf serum
FN: Fibronectin
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
G-CSF: Granulocyte-colony stimulating factor
GPx: Glutathione peroxidase
Gy: Gray
HCEC: Human corneal epithelial cells
hCMVEC: Human cerebral microvascular endothelial cells
HLEC: Human lymphatic endothelial cells
HUVEC: Human umbilical vein endothelial cells
IL: Interleukin
Ig: Immunoglobulin
IL: Interleukin
IR: Ionizing radiation
KC: Keratinocyte-derived chemokine
LDL: Low-density lipoprotein
LD-RT: Low-dose radiotherapy
LN: Laminin
LPS: Lipopolysaccharide
MCP-1: Monocyte chemoattractant protein-1
mRNA: Messenger ribonucleic acid
NFκB: Nuclear factor kappa B
RNA: Ribonucleic acid
qRT-PCR: Quantitative real-time polymerase chain reaction
PA: Plasminogen activator
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate-buffered saline
PMN: Polymorphonuclear leukocytes
RANTES: Regulated on activation, normal T cell expressed and secreted
RNA: Ribonucleic acid
RNP: Ribonucleoprotein
SOD: Superoxide dismutase 1
THBS1: Thrombospondin 1
TH2: T-helper cell subtype 2
TNF-α: Tumor necrosis factor alpha
WST-1: Water-soluble tetrazolium
XIAP: X-linked inhibitor of apoptosis protein.

Data Availability

Data supporting this study are provided in the results section or as supplementary information accompanying this paper. Further datasets used and/or analyzed during the current study are available from the authors at the University Medical Center Rostock on request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplement Figure 1: influence of low-dose irradiation on metabolic activity of murine endothelial cells, (A) without TNF-α induction and (B) with TNF-α induction. The cells were irradiated with different doses; a WST-1 assay was performed at three time points after irradiation. The extinctions were normalized to samples of 0 Gy/4 hours. Error bars present the standard deviation (±SD) from three independent experiments; wells were assayed in triplicates in each of the different experiments. Supplement Figure 2: released levels of the keratinocyte-derived chemokine (KC) in supernatant of (A) mlEND.1 and (B) bEND.3 endothelial cells. The cytokine concentration was determined by multiplex assay at two time points after irradiation with low doses of X-rays. Changes in cytokine concentrations are presented as mean (pg/mL) ± standard deviation (SD) from three independent experiments; asterisks illustrate significance: $^{*}p < 0.05$ and $^{**}p < 0.01$. Supplement Figure 3: released levels of monocyte chemoattractant protein-1 (MCP-1) in supernatant of (A) H5V and (B) mlEND.1 endothelial cells. The cytokine concentration was determined by multiplex assay at two time points after irradiation with low doses of X-rays. Changes in cytokine concentrations are presented as mean (pg/mL) ± standard deviation (SD) from three independent experiments; asterisks illustrate significance: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Supplement Figure 4: released levels of RANTES in supernatant of (A) H5V and (B) bEND.3 endothelial cells. The cytokine concentration was determined by multiplex assay at two time points after irradiation with low doses of X-rays. Changes in cytokine concentrations are presented as mean (pg/ml) ± standard deviation (SD) from three independent experiments; asterisks illustrate significance: $^{*}p < 0.05$ and $^{**}p < 0.01$. Supplement Figure 5: released levels of G-CSF in supernatant of (A) 2D- and (B) 3D-cultured endothelial cells. The cytokine concentration was determined by multiplex assay at two time points after irradiation with low doses of X-rays. Changes in cytokine concentrations are presented as mean (pg/mL) ± standard deviation (SD) from three independent experiments; asterisks illustrate significance: $^{*}p < 0.05$. *(Supplementary Materials)*

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Research Article

Inhibition of IL-17 and IL-23 in Human Keratinocytes by the A_3 Adenosine Receptor Agonist Piclidenoson

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Interleukin-17 and interleukin-23 play major roles in the inflammatory process in psoriasis. The Gi protein-associated A_3 adenosine receptor (A_3AR) is known to be overexpressed in inflammatory cells and in peripheral blood mononuclear cells (PBMCs) of patients with autoimmune inflammatory conditions. Piclidenoson, a selective agonist at the A_3AR, induces robust anti-inflammatory effect in psoriasis patients. In this study, we aimed to explore A_3AR expression levels in psoriasis patients and its role in mediating the anti-inflammatory effect of piclidenoson in human keratinocyte cells. A_3AR expression levels were evaluated in skin tissue and PBMCs derived from psoriasis patients and healthy subjects. Proliferation assay and the expression of signaling proteins were used to evaluate piclidenoson effect on human keratinocytes (HaCat). High A_3AR expression levels were found in a skin biopsy and in PBMCs from psoriasis patients in comparison to healthy subjects. Piclidenoson inhibited the proliferation of HaCat cells through deregulation of the NF-κB signaling pathway, leading to a decrease in interleukin-17 and interleukin-23 expression levels. This effect was counteracted by the specific antagonist MRS 1523. A_3AR overexpression in skin and PBMCs of psoriasis patients may be used as a target to inhibit pathological cell proliferation and the production of interleukin-17 and interleukin-23.

1. Introduction

Psoriasis, affecting 2-3% of the worldwide population, is an inflammatory, chronic skin disease considered to be an immune-mediated one. It is characterized by excessive proliferation and abnormal differentiation, accompanied by apoptosis of keratinocytes (KCs) causing erythematous, scaly patches, or plaques on the skin [1–3]. In the psoriatic skin area, expression of proinflammatory cytokines and chemokines is upregulated, attracting immune cells leading to the proliferation of local and invading cells [4]. The proinflammatory cytokine interleukin-23 (IL-23) is one of the major cytokines involved in the pathogenesis of psoriasis [5] by mediating Th17 cells, which release interleukin-17 (IL-17) into the inflamed environment. IL-23 is highly expressed in psoriatic skin and is secreted among other cells, also from KCs [6]. Its blockade by injection of specific human anti-IL-23 antibody results in neutralizing IL-23 and prevention of disease progression in psoriasis patients [7]. High levels of IL-17 have been detected in psoriatic skin lesions [8, 9], strengthening the assumption that inhibition of both cytokines will result in improvement of disease pathology.

A_3 adenosine receptor (A_3AR) is a Gi protein-coupled cell surface receptor, known to mediate anti-inflammatory effects upon activation with selective agonists. The A_3AR was found to be highly expressed in inflammatory tissues [10, 11] and in the peripheral blood mononuclear cells (PBMCs) [12–14] of patients with Crohn's disease, psoriasis, and rheumatoid arthritis (RA). The mechanism responsible for receptor overexpression suggests the involvement of the transcription factors nuclear factor kappa B (NF-κB) and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), both present in the A_3AR gene promotor and are known to be upregulated in psoriasis [12]. It was found that the inflammatory cytokine tumor necrosis factor α (TNF-α) regulates A_3AR via an autocrine pathway while an increase in this cytokine expression results in a subsequent increase in NF-κB and receptor upregulation [13].

Piclidenoson is an A_3AR highly specific agonist, inducing a robust anti-inflammatory effect demonstrated in preclinical pharmacology studies in rheumatoid arthritis, osteoarthritis, uveitis, and colitis [14–19]. This anti-inflammatory effect is mediated via downregulation of the NF-κB signaling pathway resulting in TNF-α inhibition [14, 16].

The efficacy of piclidenoson in plaque psoriasis has been demonstrated in phase II clinical studies [15, 20].

In the present study, we have further investigated the expression of the A_3AR in cells from psoriasis patients and explored the utilization of A_3AR as a therapeutic target.

2. Materials and Methods

2.1. Reagents. The A_3AR agonist 1-deoxy-1-[6-[[(iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-(-D-ribofuranuronamide), piclidenoson (also known as IB-MECA), was synthesized by Can-Fite BioPharma. A stock solution of 10 mM was prepared in dimethyl sulfoxide (DMSO) and further diluted in phosphate-buffered saline (PBS). The A_3AR antagonist MRS 1523 was purchased from Sigma (St. Louis, MO, USA) and diluted in the same manner as for piclidenoson. Dulbecco's Modified Eagle's medium (DMEM) 4.5 g/L glucose, fetal bovine serum (FBS), and antibiotics for cell cultures were obtained from Beit HaEmek, Haifa, Israel. Polyclonal antibodies against human A_3AR, phosphoinositide 3-kinase (PI3K), NF-κB, TNF-α, IL-17, IL-23, and β-actin were purchased from Santa Cruz Biotechnology Inc., Ca, USA. Monoclonal antibody against human p-AKT was purchased from Cell Signaling Technology, MA, USA.

2.2. Patients and Healthy Subject Population. To analyze A_3AR expression levels, blood samples were collected from 48 psoriasis patients with moderate to severe disease that participated in a phase 2 study as part of the protocol (NCT00428974) [5]. Blood samples from 50 healthy subjects were collected upon ethical committee approval. All donors signed an informed consent prior to blood withdrawal. To separate PBMCs, the blood (16 ml) was collected in BD Vacutainer® CPT tubes (BD, Franklin Lakes, NJ, USA) and centrifuged according to the manufacturer's instructions and washed with PBS.

2.3. RT-PCR Analysis of Formalin-Fixed Paraffin-Embedded Skin Biopsy. RNA expression levels in psoriasis formalin-fixed paraffin-embedded skin biopsy were detected as previously described [21]. In brief, the formalin-fixed, paraffin-embedded skin biopsy tissue was purchased from a tissue bank. To differentiate between the psoriasis tissue and normal skin regions, slides were stained with H&E and observed by a pathologist. Later on, nonstained sequential slides were marked for psoriasis and normal area based on the stained slides.

Tissue sections (20 μm) were mounted on slides that were stained by hematoxylin and eosin (H&E) and were observed by a pathologist. Tissue sections on slides were deparaffinized in xylene and rehydrated by washing in serial dilutions of ethanol. Slides were used immediately or stored at −80°C until used. After rehydration, 20 μl of solution A [1.25X PCR buffer (200 mM Tris–HCl and 500 mM KCl), 6.25 mM MgCl2, 5 units RNasin (Promega, Madison, WI, USA), 2 mM DTT, and 1 unit RQ1 RNase-free DNase (Promega)] was applied directly to the marked area. The psoriatic skin lesion and the adjacent tissue were completely scraped off the slide using a pipette tip and were collected to a different microcentrifuge tube. The samples were treated with proteinase K at a final concentration of 0.1 mg/ml. The samples were incubated at 37°C for 1 hour to allow for DNA digestion. Cells lysate were heated to 95°C for 15 min to inactivate DNase and proteinase K. After centrifugation at 14,000 rpm for 5 min, 17 μl of the supernatant was transferred to a separate tube, and 4 μl of reverse transcription mixture [5 mM deoxynucleoside triphosphate, 2.5 μM random hexamer, 5 units RNasin, 100 units SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen), and the primers for A_3AR 5′-ACGGTGAGGTACCACAGCTTGTG and 3′ ATACCG CGGGATGGCAGACC] was added. The reverse transcription reaction was performed at 45°C for 45 min followed by heating to 99°C for 5 min. Next, 50 cycles of 94°C for 30 s, 59°C for 45 s, and 73°C for 45 s were performed. Products were electrophoresed on 2% agarose gels, stained with ethidium bromide (Et-Br), and visualized with UV illumination. The specificity of the RT-PCR reaction was confirmed by size determination on agarose gels in comparison with a positive control, from RNA extracted using standard techniques, and by sequencing the RT-PCR product and comparing the sequences to that of the known sequences (ADORA3-L77729, L77730). The absorbance of the bands (Et-Br) was quantified using an image analysis system. To quantitate A_3AR mRNA expression, the absorbance value was normalized against the cell number in each psoriatic lesion or healthy skin tissue.

2.4. Cell Culture. HaCat cells derived from human KCs (AddexBio, San Diego, CA, USA) were grown in DMEM 4.5 g/L glucose medium containing penicillin, streptomycin, 2 mM L-glutamine, and 10% FBS. The cells were maintained in T-75 flasks at 37°C in a 5% CO^2 incubator and transferred to a freshly prepared medium twice weekly. For all studies, serum-starved cells were used. FBS was omitted from the cultures for 18 hours, and the experiment was carried out on monolayers of cells in DMEM 4.5 g/L glucose medium supplemented with 1% FBS in a 37°C and 5% CO^2 incubator.

2.5. [^{3}H]-Thymidine Incorporation Assay. [^{3}H]-Thymidine incorporation assay was used to evaluate cell growth. HaCat cells (2500 cells/well) were incubated with piclidenoson 10 nM and MRS 1523 (50 nM) in 96-well plate for 48 hours. For the last 24 hours of incubation, each well was pulsed with 1 mCi [^{3}H]-thymidine. Cells were harvested, and the [^{3}H]-thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ, USA). These experiments were repeated at least 4 times.

2.6. Western Blot Analysis. To detect expression levels of A_3AR, PI3K, p-AKT, NF-κB, TNF-α, IL-17, IL-23, and β-actin, the protein extract from piclidenoson-treated or -untreated HaCat cells was utilized. Cells were incubated in

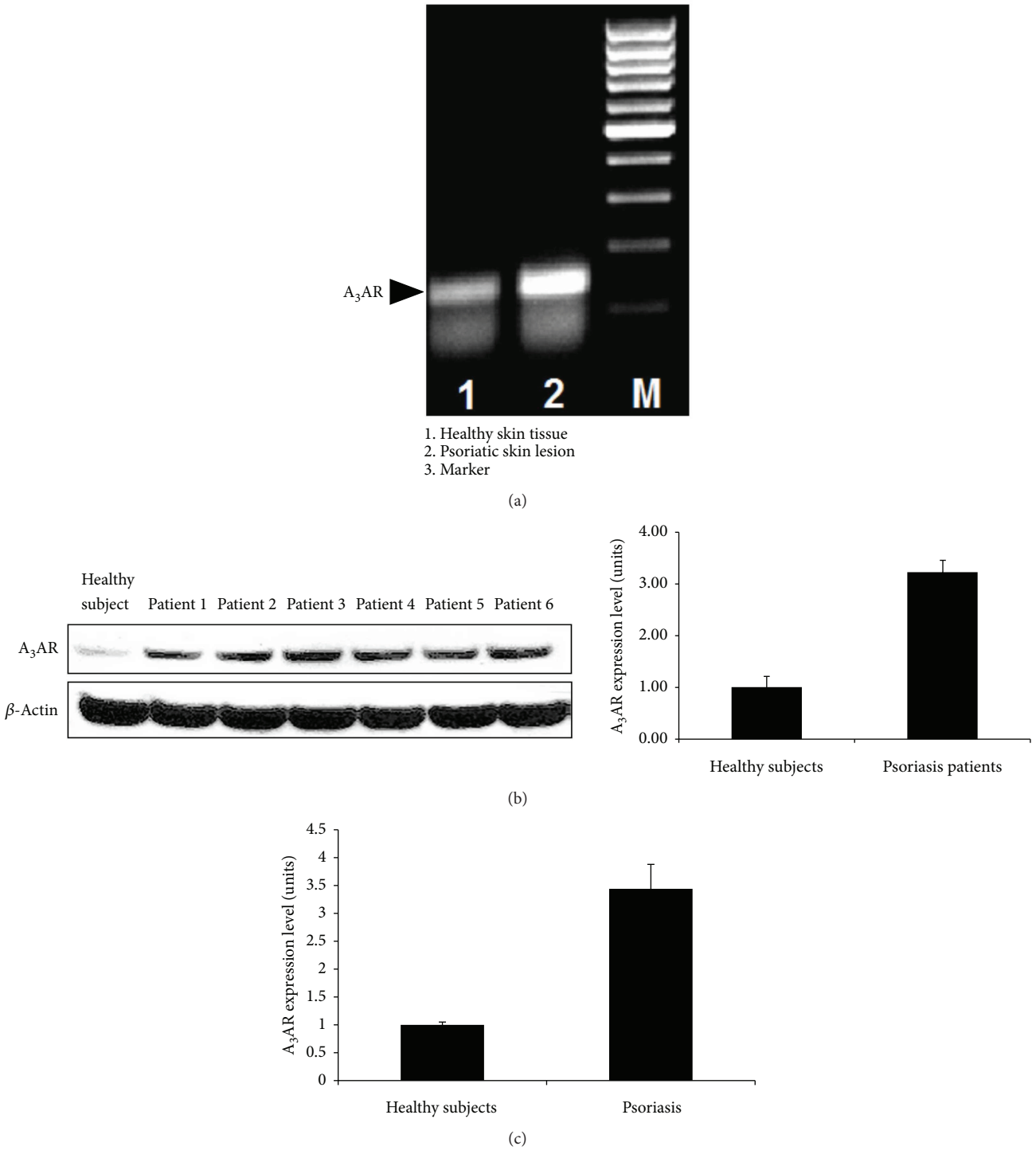

Figure 1: A_3AR overexpression in a skin lesion and PBMCs from psoriasis patients. (a) A_3AR expression level was evaluated in formalin-fixed paraffin-embedded representative skin lesion from psoriasis patient. A_3AR mRNA in the psoriatic skin lesion was compared to adjacent healthy skin tissue using RT-PCR. A_3AR was highly expressed in the skin lesion compared to the healthy tissue. (b) A_3AR protein expression level was analyzed using WB analysis in PBMCs from psoriasis patients ($n = 48$) compared with healthy subjects ($n = 50$). A significant ($p = 0.0001$) A_3AR upregulation is noted. (c) A_3AR mRNA expression level was tested in PBMCs from psoriasis patients compared to healthy subjects using real-time PCR.

the presence and absence of piclidenoson (10 nM) for 48 hours at 37°C. At the end of the incubation period, cells were then rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50 mM Tris buffer pH = 7.5, 150 mM NaCl, NP 40 0.5% for 20 min). Cell debris was removed by centrifugation for 10 min, at 7500 ×g. The supernatant was utilized for Western blot analysis. Protein concentrations were determined using the NanoDrop (Thermo Scientific).

Equal amounts of the sample (50 μg) were separated by SDS-PAGE, using 4–12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitrocellulose membranes (Pall Corporation, Florida, USA). Membranes were blocked with 5% bovine serum albumin and incubated with the desired primary antibody (dilution 1 : 1000) for 24 hours at 4°C. Blots were then washed and incubated with a secondary antibody for 1 hour at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison, WI, USA). Densitometry of protein expression was normalized against β-actin and expressed as % of control.

2.7. A_3AR mRNA Expression Level Analysis. For quantification of A_3AR levels from psoriasis patients, blood samples were withdrawn to BD Vacutainer CPT™ tube with sodium citrate. Cells were separated according to the manufactory protocol. Total RNA was extracted using the RNeasy mini kit (QIAGEN) with QIAshredder Spin Columns (QIAGEN). RNA (500 ng) was reverse transcribed by high-capacity cDNA reverse transcription kit (AB Applied Biosystems) according to the manufactory protocol.

Real-time PCR was performed using rotor gene 3000 RT-PCR detection system (Corbett Research, Australia) according to manufacturer's instruction. Briefly, The PCR primer used was 5′- GGCCAATGTTACCTACATCAC -3′ [forward] and 5′-CAGGGCTAGAGAGACAATGAA-3′ [reversed] for ADORA3. Processes were set as follows: initial denaturation at 95°C for 10 min, 30 cycles of amplification including a denaturation step at 95°C for 15 sec, an annealing step at 50°C, and an extension step at 72°C for 15 sec. Direct detection of PCR products monitored by measuring the fluorescence produced by the result of TaqMan probe hydrolysis after every cycle.

2.8. Statistical Analysis. The results were evaluated using the Student's *t*-test and analysis of variance (ANOVA) test with statistical significance set at $p < 0.05$. Comparison between the mean values of different experiments was carried out. All data are reported as mean ± SD.

3. Results

3.1. A_3AR Is Overexpressed in Skin Tissue and PBMCs of Psoriasis Patients. High expression levels of A_3AR were found in a representative psoriatic skin lesion compared to the normal adjacent tissue within the skin biopsy (Figure 1(a)). High A_3AR protein expression level was found in 97% of 48 PBMCs samples of psoriasis patients in comparison to 50 samples from healthy subjects. Figure 1(b) is a representative blot from 6 patients versus healthy subject showing an increase of ~3.4 fold in A_3AR expression levels compared to the naive ($p < 0.0001$). Overexpression of A_3AR mRNA was also detected in the patients' PBMCs showing an increase of ~3.5 fold compared to that of the healthy subjects (Figure 1(c)).

3.2. Piclidenoson Inhibits the Proliferation of HaCat Cells. Piclidenoson inhibits the proliferation of HaCat cells by 40% ±8.1. Introduction of the antagonist MRS 1523 to the culture system counteracted piclidenoson inhibitory effect indicating that the response was A_3AR mediated ($p < 0.01$) (Figure 2).

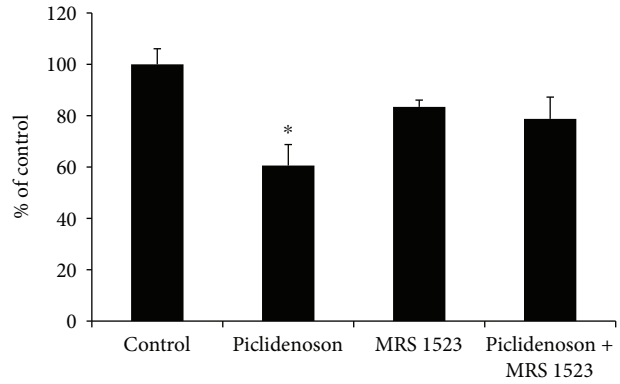

Figure 2: Piclidenoson inhibits the proliferation of HaCat cells. Proliferation assay was performed on HaCat cells that were incubated in the presence of piclidenoson (10 nM) and A_3AR antagonist MRS 1523 (50 nM) for 48 hours ($^*p < 0.05$).

3.3. Piclidenoson De-Regulates Cell Growth Regulatory Proteins. Piclidenoson induced a decrease in A_3AR expression level, demonstrating a response to the agonist. The specificity of this response was shown by the antagonist MRS 1523 which counteracted this effect (Figure 3). Downstream to receptor activation, a decrease in the expression level of PI3K, p-AKT, NF-κB, and TNF-α took place (Figure 4). Based on our former experience, the total AKT has not been modulated upon treatment with piclidenoson in inflammatory cells [14].

3.4. Piclidenoson Inhibits IL-17 and IL-23. Piclidenoson inhibits IL-17 and IL-23 expression levels whereas MRS 1523 reversed the inhibitory effect (Figure 5).

4. Discussion

In this study, we further demonstrate the overexpression of A_3AR in a skin lesion and in the PBMCs of psoriasis patients with moderate to severe disease. Targeting the receptor with the highly specific agonist piclidenoson, A_3AR induced the inhibition of HaCat cell proliferation, a spontaneously immortalized human epithelial line that maintains full epidermal differentiation capacity and has been widely used as an *in vitro* model for the study of psoriasis [22, 23]. Moreover, piclidenoson induced a decrease in the expression level of PI3K, p-AKT, NF-κB, TNF-α, IL-17, and IL-23, known to act as potent inflammatory mediators in psoriasis.

It has been reported earlier that A_3AR is overexpressed in the PBMCs of patients with autoimmune inflammatory conditions such as crohn's disease, rheumatoid arthritis, and psoriasis, compared to healthy subjects [12]. Interestingly, A_3AR overexpression was found to be directly correlated to NF-κB and CREB, two transcription factors present in the A_3AR gene promoter. NF-κB is one of the most important mediators in the pathogenesis of psoriasis. It is a transcription factor which belongs to the Rel1 family and is involved

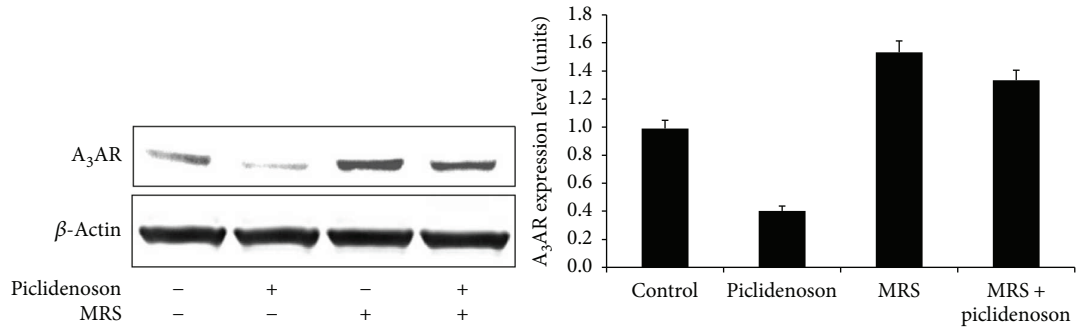

Figure 3: Piclidenoson downregulates A_3AR protein expression level. HaCat cells were incubated in the presence and absence of piclidenoson and MRS 1523 for 48 hours. Downregulation of A_3AR was demonstrated upon piclidenoson treatment and reversed by the introduction of MRS 1523 to the culture.

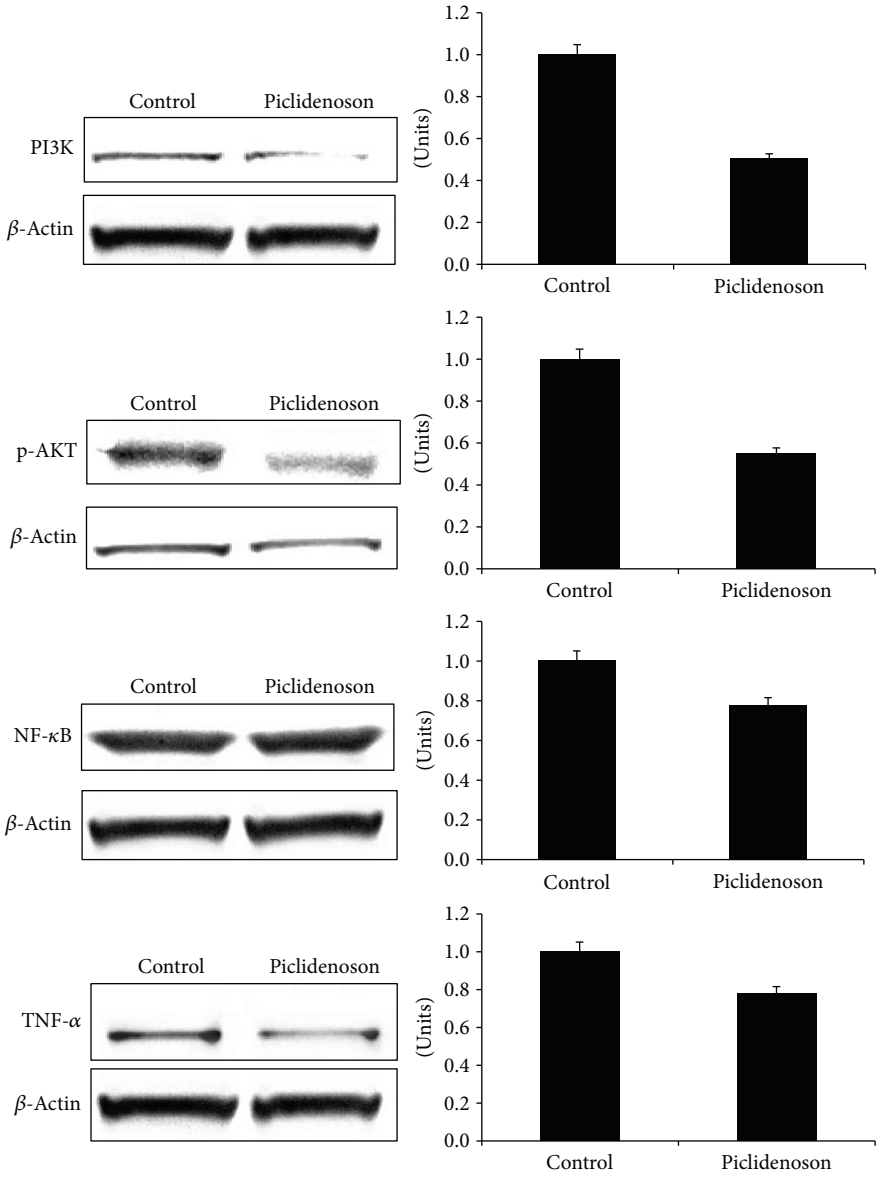

Figure 4: Piclidenoson downregulates the NF-κB signaling pathway. HaCat cells were incubated in the presence and absence of piclidenoson (10 nM) for 48 hours. The expression level of PI3K, p-AKT, NF-κB, and TNF-α was tested by Western blot analysis.

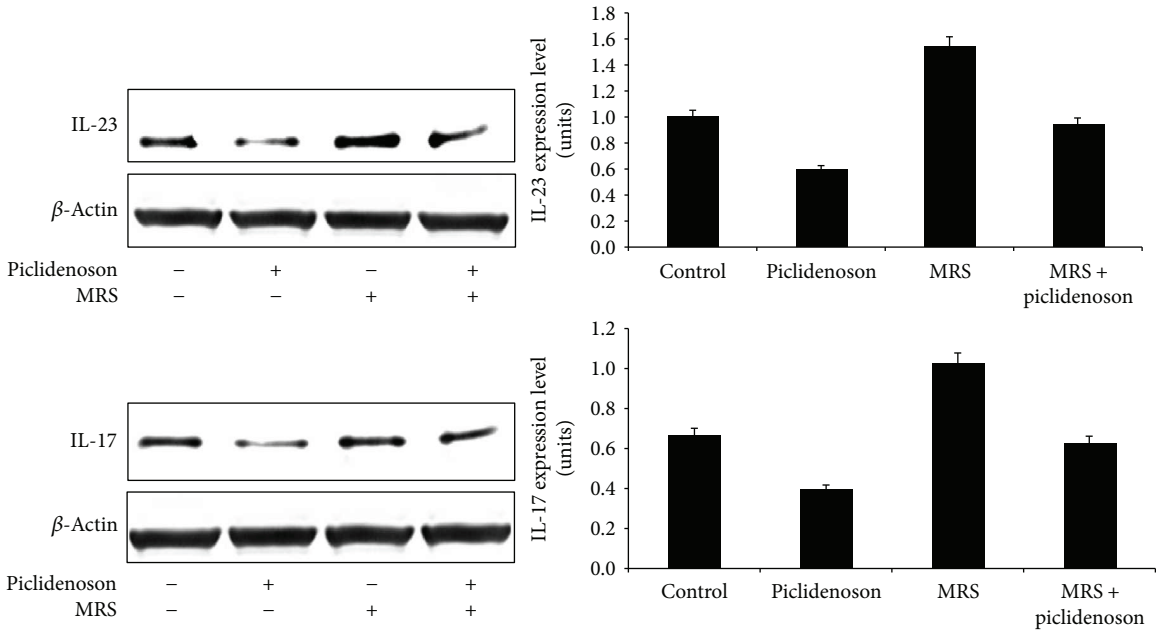

Figure 5: Piclidenoson downregulates IL-17 and IL-23. HaCat cells were incubated in the presence and absence of piclidenoson (10 nM) and MRS 1523 (50 nM) for 48 hours. The expression level of the inflammatory cytokines IL-17 and IL-23 was tested by Western blot analysis.

in the regulation and function of different proinflammatory genes. It has been found that many of the triggering factors for psoriasis evolve via the activation of NF-κB which subsequently translocate to the epidermis and basal cells, resulting in epidermal hyperplasia and inflammation, thereby affecting KCs proliferation and differentiation [22]. The involvement of a large surface skin area in psoriasis patients intensifies the role of factors such as NF-κB and TNF-α, leading to amplification of the inflammatory process, which may also attribute to A_3AR overexpression.

A_3AR was found to be overexpressed in the psoriasis skin lesion biopsy in comparison to the normal adjacent tissue. Also, mRNA A_3AR expression levels were ~3.5 fold higher in the PBMCs of the psoriasis subjects versus healthy subjects. These findings corroborate with former data showing that high A_3AR expression in the inflammatory target organ is mirrored in the patients' PBMCs [12].

A support for the utilization of the A_3AR as a therapeutic target came from the data of the present study showing that piclidenoson inhibited the proliferation of HaCat cells.

In the keratinocyte cultures, shortly after A_3AR activation with piclidenoson, receptor protein expression was downregulated, a response known to initiate downstream molecular events leading to cell proliferation inhibition [10]. This downregulation was mostly reversed by introducing the antagonist MRS 1523 to the culture, indicating that piclidenoson effect is A_3AR mediated. Receptor downregulation is a general mechanism typical of Gi protein receptors. This family of receptors responds to ligand activation by receptor internalization (to the cytosol), degradation, resynthesis, and recycling to the cell surface [24]. During these events, receptor desensitization/resensitization takes place and different signaling pathways are initiated [25]. We suggest that the downregulation of receptor expression in this study represents the rapid response of the HaCat cells to agonist stimulation and the initiation of downstream signaling events.

Subsequent to the deregulation of NF-κB and related proteins, a decrease in the expression levels of TNF-α, IL-17, and IL-23 took place, further strengthening the utilization of piclidenoson as a drug candidate to combat psoriasis. By adding MRS 1523 to the culture system, the inhibition of IL-17 and IL-23 by piclidenoson was abolished suggesting that A_3AR acts as a modulator for these cytokines. Activation of NF-κB and TNF-α induces IL-23 production [26, 27], therefore suggesting that the inhibitory effect of piclidenoson on NF-κB and TNF-α may result in deregulation of IL-23 and IL-17. Monoclonal antibodies against TNF-α, IL-17, and IL-23 were found to mediate a strong anti-inflammatory effect that has been already implemented for the treatment of psoriasis patients and proved to be highly efficacious [25, 27–31].

5. Conclusion

Piclidenoson, a selective A_3AR agonist, inhibits KCs proliferation via the deregulation of the NF-κB signal transduction pathway resulting in a decrease in the expression levels of IL-17 and IL-23. Piclidenoson has previously been tested in 2 phase II clinical studies showing an anti-inflammatory effect and good safety profile [15, 32]. The current data further support the development of this drug candidate for the treatment of psoriasis.

Abbreviations

Kcs: Keratinocytes
IL-23: Interleukin-23
IL-17: Interleukin-17

A_3AR: A_3 adenosine receptor
PBMCs: Peripheral blood mononuclear cells
RA: Rheumatoid arthritis
NF-κB: Nuclear factor kappa B
CREB: cAMP response element-binding protein
TNF-α: Tumor necrosis α
PI3K: Phosphoinositide 3-kinase.

Disclosure

Shira Cohen and Michael H. Silverman are consultants at Can-Fite BioPharma. Faina Barer and Inbal Itzhak are employees at Can-Fite BioPharma. Pnina Fishman is an executive of Can-Fite BioPharma and has shares and stock options. The authors alone are responsible for the content and writing of the paper.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Shira Cohen, Michael H. Silverman, and Pnina Fishman designed the studies, interpreted the data, and wrote the manuscript. Faina Barer, Inbal Itzhak, and Shira Cohen performed the experiments. All authors have revised and approved the final version of the manuscript.

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Research Article

Prevention of Cyclophosphamide-Induced Immunosuppression in Mice with the Antimicrobial Peptide Sublancin

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Sublancin is a glycosylated antimicrobial peptide produced by *Bacillus subtilis* 168 with combined antibacterial and immunomodulatory activities. The purpose of this study was to evaluate the protective effects of sublancin on immunosuppression in cyclophosphamide-treated mice. In normal mice, the phagocytic activity of mouse peritoneal macrophages was significantly enhanced by oral administration of sublancin (1.0 mg/kg body weight) to BALB/c mice for 7 days ($P < 0.01$). In addition, the mRNA expression of IL-1β, IL-6, and TNF-α in peritoneal macrophages from sublancin- (1.0 mg/kg body weight) administered mice was significantly increased ($P < 0.05$). In cyclophosphamide-treated mice, oral sublancin administration accelerated the recovery of peripheral white blood cells, red blood cells, hemoglobins, and platelets and enhanced the macrophage phagocytic activity. Furthermore, sublancin restored the mRNA levels of IL-2, IL-4, and IL-6 in the spleen. Finally, the intestinal absorption of sublancin was poor as detected in the Caco-2 transwell system. Taken together, these findings suggest that sublancin plays a crucial role in the protection against immunosuppression in cyclophosphamide-treated mice and could be a potential candidate for use in immune therapy regimens.

1. Introduction

Cyclophosphamide (Cy) is a major constituent of cancer chemotherapy agent and widely used in the treatment of various types of cancer [1]. Unfortunately, immunosuppression induced by Cy increases incidence of secondary infections and mortality, which is a major limiting factor in clinical chemotherapy [2]. Therefore, many attempts are being investigated to obtain immunomodulatory agents that can reduce the cytotoxic side effects and enhance immunity in chemotherapy-treated patients.

Antimicrobial peptides (AMPs) are a variety of naturally short-amino-acid-chain molecules that provide immediately effective and nonspecific defenses against invading pathogens [3]. Emerging evidence suggests that AMPs are involved in the modulation of the immune response [4–6]. Sublancin is a 37-amino acid AMP produced by *Bacillus subtilis* 168 with high stability [7]. In addition to direct antibacterial activity, sublancin has been reported to possess immunomodulatory activity [8]. Our previous study indicated that sublancin ameliorated *Clostridium perfringens*-induced necrotic enteritis in broilers via alleviating inflammatory reaction [9]. In addition, it was demonstrated that sublancin attenuated the intestinal inflammation induced by methicillin-resistant *Staphylococcus aureus* (MRSA) in mice through inhibition of NF-κB activation [8]. Moreover, we found

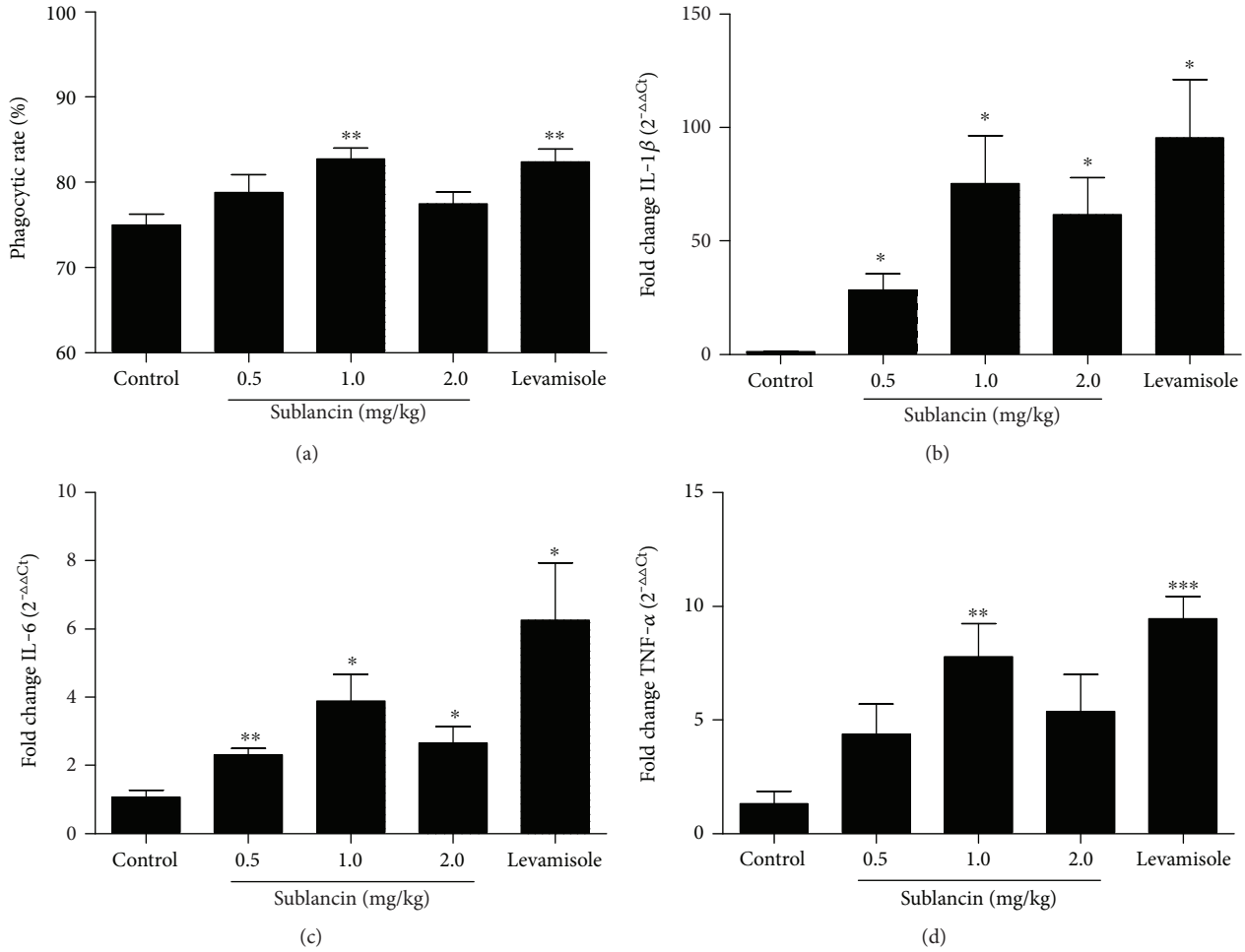

Figure 1: Effect of oral administration of sublancin on the phagocytic activity (a) and gene expression of cytokines (b–d) in P-Mac ex vivo. Six-week-old female BALB/c mice were separated into five groups and orally administered with sublancin (0.5, 1.0, and 2.0 mg/kg body weight/day) or 2.5 mg/kg levamisole hydrochloride (positive control) for 7 days. The vehicle control group was administered with distilled water daily. Mouse P-Mac were isolated and cultured in 10% FBS DMEM. After cultivation, the cells were used to analyze the phagocytic activity and gene expression of cytokines, respectively. Values are expressed as means ± SEM ($n = 6$); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with vehicle control group.

that macrophages are critical for the protective effect of sublancin in a mouse model of MRSA-induced sublethal infection (unpublished data).

However, the immunomodulatory effect of sublancin on immunosuppressed mice remains poorly understood. Additionally, whether AMPs can be absorbed by the intestine is rarely reported. The objective of this study was to elucidate the protective effects of sublancin on immunosuppression in Cy-treated mice. In this study, we (i) investigated the effects of oral administration of sublancin on peritoneal macrophages in vivo, (ii) evaluated the protective effects of sublancin in a mouse immunosuppression model, and (iii) studied the intestinal absorption of sublancin in the Caco-2 transwell system as well as in the in vivo biodistribution of sublancin.

2. Materials and Methods

2.1. Animals. Female BALB/c mice of 6–8 weeks old were purchased from HFK Bioscience Co. Ltd. (Beijing, China). The mice were housed in plastic cages under 12 h light/dark cycle and were provided with food and water ad libitum. All experimental procedures were approved by the China Agricultural University Institutional Animal Care and Use Committee (ID: SKLAB-B-2010-003).

2.2. Effects of Oral Administration of Sublancin on Peritoneal Macrophages In Vivo

2.2.1. Experimental Design. Sublancin was produced in our laboratory using a highly efficient expression system involving *Bacillus subtilis* 800. The amino acid sequence of sublancin was GLGKAQCAALWLQCASGGTIGCGG GAVACQNYRQFCR, and the peptide purity was >99.6% as determined by high-performance liquid chromatography. Sublancin was produced as lyophilized powder and stored at −20°C until use. Six-week-old female BALB/c mice ($n = 6$) were orally administered with sublancin at 0.5, 1.0, or 2.0 mg/kg body weight (BW)/d for 7 consecutive days. Mice in the control group were administrated

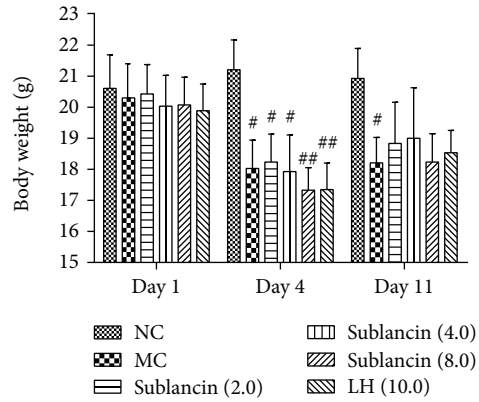

Figure 2: Effects of sublancin on the body weight in cyclophosphamide-treated mice. NC (normal control) = mice were treated with saline; MC (model control) = mice were administered with cyclophosphamide 80 mg/kg/d via i.p. injection for 3 days and gavaged with saline for 7 days; sublancin (2.0, 4.0, and 8.0) = mice were administered with cyclophosphamide 80 mg/kg/d via i.p. injection for 3 days and gavaged with sublancin at 2.0, 4.0, or 8.0 mg/kg/d for 7 days, respectively; LH (10.0) = mice were administered with cyclophosphamide 80 mg/kg/d via i.p. injection for 3 days and gavaged with levamisole hydrochloride for 7 days. Values are expressed as means ± SEM ($n = 9$); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with NC group.

with sterile saline daily. Mice in the positive control group were given 2.5 mg/kg BW/d levamisole hydrochloride (LH) in a similar manner as the sublancin treatment. On day 8, peritoneal macrophages (P-Mac) were collected as previously described [10] and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Peritoneal macrophages were harvested for phagocytosis assay and determining the gene expression levels of cytokines by real-time PCR.

2.2.2. Phagocytosis Assay. Phagocytosis was determined by flow cytometry as described previously [11]. Briefly, peritoneal macrophages from sublancin-administered mice were cultured in 6-well plates (1×10^6 cells/well). After cultivation for 12 h, 100 μL of suspended fluorescent microspheres in PBS was added to the wells (cell-to-bead ratio 1 : 20) and the cells were incubated at 37°C for 1 h. Phagocytosis was terminated by the addition of 2 mL ice-cold PBS, and then the cells were washed three times with cold PBS and harvested. Flow cytometric analysis was carried out using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, San Jose, CA, USA).

2.2.3. Quantitative Real-Time PCR. Peritoneal macrophages were inoculated in supplemented DMEM (10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) into 6-well plates at 1×10^6 cells/well and cultured for 12 h. Then, total RNA was extracted from the cell pellet using the TRIzol reagent (Invitrogen, Carlsbad, CA). The quality and quantity of total RNA were determined by gel electrophoresis and a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The first-strand cDNA was synthesized from the extracted RNA (1 μg) using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Otsu, Japan) according to the manufacturer's instructions. Real-time PCR was performed on a LightCycler (Roche) with SYBR Green PCR Master Mix (Takara, Otsu, Japan). The relative amounts of mRNAs were normalized against GAPDH and analyzed using the $2^{-\Delta\Delta Ct}$ method [12]. The primer sequences used were as follows: IL-1β forward, 5′-GCCT TGGGCCTCAAAGGAAAGAATC-3′; IL-1β reverse, 5′-GG AAGACACAGATTCCATGGTGAAG-3′; IL-6 forward, 5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′; IL-6 reverse, 5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′; TNF-α forward, 5′-CCTCCCTCTCATCAGTTCTATGG-3′; TNF-α reverse, 5′-CGTGGGCTACAGGCTTGTC-3′; GAPDH forward, 5′-ACCCCAGCAAGGACACTGAGCAAG-3′; and GAPDH reverse, 5′-ACCCCAGCAAGGACACTGA GCAAG3′.

2.3. Cyclophosphamide-Induced Immunosuppression in Mice

2.3.1. Experimental Model and Treatment Protocols. Eight-week-old female BALB/c mice were randomly assigned to 6 groups consisting of 9 mice each. Mice in the normal control (NC) group were treated once daily with sterile saline for 10 consecutive days. From days 1 to 3, the other five groups of mice were administered with cyclophosphamide at 80 mg/kg BW/d via intraperitoneal injection. From days 4 to 10, the mice were given the following treatments: the model control (MC) group was gavaged with 0.2 mL sterile saline; the three sublancin groups were gavaged with 0.2 mL sublancin at 2.0, 4.0, and 8.0 mg/kg BW/d; and the positive control group was gavaged with 0.2 mL levamisole hydrochloride (LH) at 10 mg/kg BW/d. Levamisole hydrochloride is an agent that has been previously used as an antihelminthic drug in clinical application. In addition, the immunoenhancing effects of levamisole hydrochloride have been demonstrated by many studies [13, 14]. We chose levamisole hydrochloride as a positive control in a cyclophosphamide-induced immunosuppressed mouse model according to several similar previous studies [15, 16]. Body weight of each animal was measured on days 1, 4, and 11. Twenty-four hours after the last drug administration, the animals were killed by cervical dislocation. Three of the animals in each group were used for the carbon clearance test, and the other six were used for other experiments as described below.

2.3.2. Carbon Clearance Test. The macrophage phagocytic function was assessed via the carbon clearance test. On day 11, mice were injected with diluted India ink through the tail vein, at a concentration of 100 μL/10 g body weight. Blood samples were collected from the retinal venous plexuses at 2 min (t_1) and 10 min (t_2) after the injection, and 20 μL blood was then mixed immediately with 2 mL 0.1% Na_2CO_3. The absorbance at 600 nm was measured on a spectrophotometer with 0.1% Na_2CO_3 as the blank. The liver and the spleen were weighted, and the rate of carbon

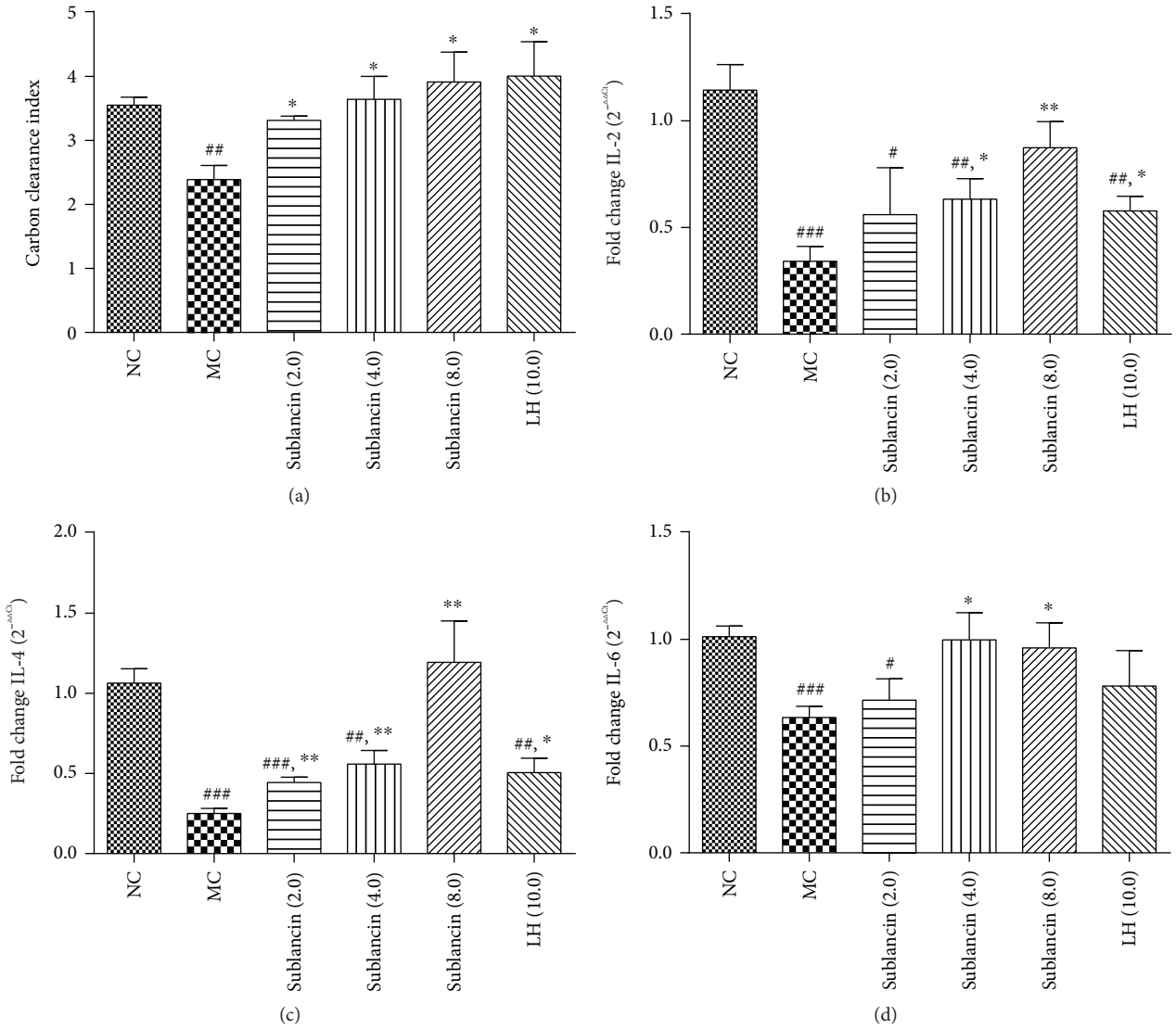

Figure 3: Effect of sublancin on the macrophage phagocytic index employing the carbon clearance test (a) and cytokine mRNA expression in the spleen of immunosuppressed mice (b–d). NC (normal control) = mice were treated with saline; MC (model control) = mice were administered with cyclophosphamide 80 mg/kg/d via i.p. injection for 3 days and gavaged with saline for 7 days; sublancin (2.0, 4.0, and 8.0) = mice were administered with cyclophosphamide 80 mg/kg/d via i.p. injection for 3 days and gavaged with sublancin at 2.0, 4.0, or 8.0 mg/kg/d for 7 days, respectively; LH (10.0) = mice were administered with cyclophosphamide 80 mg/kg/d via i.p. injection for 3 days and gavaged with levamisole hydrochloride for 7 days. Values are expressed as means ± SEM of three (a) or six (b–d) animals. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with NC group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with MC group.

clearance (K) as well as the carbon clearance index (α) was calculated by the following formulas: $K = (\lg \mathrm{OD}_1 - \lg \mathrm{OD}_2)/(t_2 - t_1)$, $\alpha = \sqrt[3]{K} \times \text{body weight}/(\text{liver weight} + \text{spleen weight})$, $t_2 = 10$ min, and $t_1 = 2$ min, where OD_1 and OD_2 are the absorbance values at 2 min and 10 min, respectively.

2.3.3. Peripheral Hemogram Analysis. Blood was collected into clean tubes with ethylenediaminetetraacetic acid (EDTA) by extracting the eyeballs on the day of sacrifice. Peripheral hemogram analysis was carried out using a Coulter LH755 Hematology Analyzer, which included white blood cells (WBC), red blood cells (RBC), hemoglobins (HGB), and platelets.

2.3.4. Measurement of Cytokine mRNAs in the Spleen. Twenty-four hours after the last administration, six mice from each group were sacrificed via cervical dislocation, and their spleens were aseptically removed. Total RNA was extracted, and the levels of mRNA expression of IL-2, IL-4, and IL-6 were evaluated by real-time PCR as described above. The following primers were used: IL-2, forward 5′-AGGAAC CTGAAACTCCCCAG-3′ and reverse 5′-AAATCCAGAAC

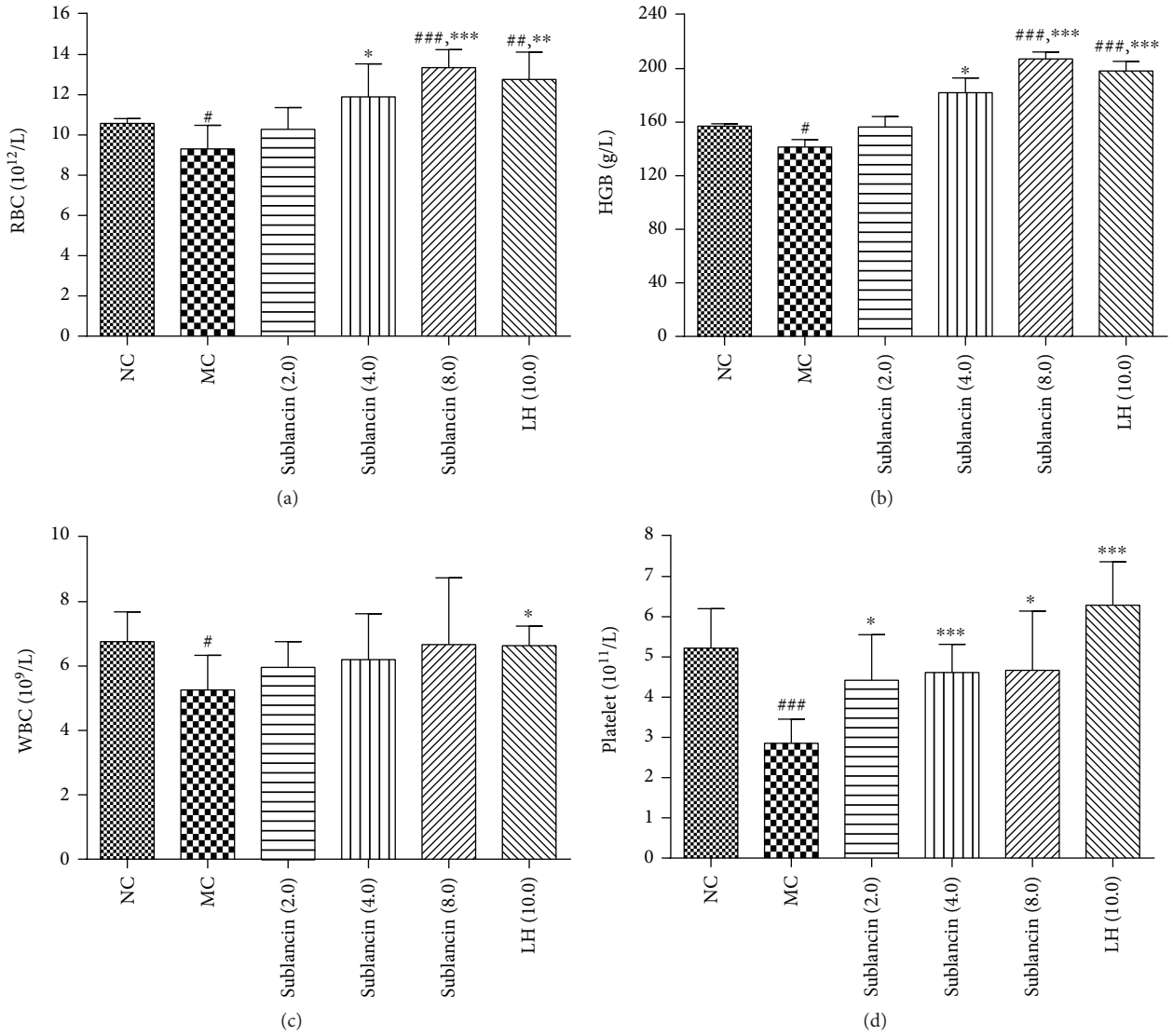

Figure 4: Effects of sublancin on numbers of red blood cells (RBC, 10^{12}/L) (a), hemoglobin (HGB, g/L) (b), white blood cells (WBC, 10^9/L) (c), and platelet (10^{11}/L) (d) in cyclophosphamide-treated mice. NC (normal control) = mice were treated with saline; MC (model control) = mice were administered with cyclophosphamide 80 mg/kg/d via i.p. injection for 3 days and gavaged with saline for 7 days; sublancin (2.0, 4.0, and 8.0) = mice were administered with cyclophosphamide 80 mg/kg/d via i.p. injection for 3 days and gavaged with sublancin at 2.0, 4.0, or 8.0 mg/kg/d for 7 days, respectively; LH (10.0) = mice were administered with cyclophosphamide 80 mg/kg/d via i.p. injection for 3 days and gavaged with levamisole hydrochloride for 7 days. Values are expressed as means ± SEM ($n = 6$); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with NC group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with MC group.

ATGCCGCAG-3′, and IL-4, forward 5′-TCTCGAATGTA CCAGGAGCC-3′ and reverse 5′-ACCTTGGAAGCCCT ACAGAC-3′.

2.4. Caco-2 Cell Culture. The human colon carcinoma cell line Caco-2 was purchased from the American Type Culture Collection (Rockville, MD) and cultured in DMEM supplemented with 10% FBS, 1% nonessential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained in a humidified incubator at 37°C, with an atmosphere containing 5% CO_2. For the transport experiment, subconfluent cells were seeded at a density of 4×10^5 cells per well onto microporous membranes inserted into transwells (0.4 μm pores, 1.13 cm^2, Corning). The inserts were placed in 12-well plates with 0.5 mL of medium in the apical compartment and 1.5 mL medium in the basolateral compartment. The medium was replaced every other day.

2.5. Transport Experiments. The transport experiments were performed 21 days after seeding in medium. The integrity of the Caco-2 cell monolayer was confirmed by examining transepithelial electrical resistance (TEER) using an epithelial tissue voltohmmeter (World Precision Instruments Inc., Florida, USA). Only inserts that exceeded a resistance of 400 $\Omega \cdot cm^{-2}$ were used, and the transport experiments were performed with a concentration of 200 μM of sublancin in the apical compartment. After 0, 0.5, 1, 2, and 3 h, 100 μL

Table 1: Summary of the analysis of sublancin through a Caco-2 monolayer.

Incubation time (h)	C_{apical} (μM)	$C_{basolateral}$ (μM)	P_{app} (10^{-6} cm·s^{-1})
0.5	147.4 ± 1.48	<LOD	—
1	135.7 ± 3.37	3.78 ± 1.02	7.00 ± 0.25
2	113.5 ± 4.20	9.56 ± 2.21	7.65 ± 0.69
3	112.0 ± 2.27	23.59 ± 1.96	9.58 ± 0.58

The concentrations determined by high-performance liquid chromatography are shown as mean ± SEM ($n = 6$). Based on these data, the transport rate P_{app} was calculated.

samples was taken from the basolateral side and 50 μL from the apical side. All samples were stored at −20°C prior to analysis by high-performance liquid chromatography [17]. The apparent permeability coefficient (P_{app} in cm/s) was calculated by the formula:

$$P_{app} = \frac{\Delta c \cdot V}{\Delta t \cdot c_0 \cdot A}, \quad (1)$$

where Δc is the sublancin concentration (μM) in the receiver compartment, V is the volume of the receiver compartment, Δt is the duration of the transport experiment (s), c_0 is the initial concentration in the donor compartment (μM), and A is the surface area of the membrane (cm^2).

The cytotoxic effect of sublancin on Caco-2 cells was assayed using the cell counting kit-8 (Sigma-Aldrich). Sublancin showed no cytotoxicity up to a concentration of 1600 μM within 24 h [8].

2.6. In Vivo Fluorescence Imaging. To observe the biodistribution of sublancin in vivo, sublancin was labeled with the near-infrared dye Cy7 monoreactive NHS ester (Invitrogen, CA, USA). Female BALB/c mice of 6–8 weeks old were given an oral dose of 100 μL Cy7-stained sublancin or free Cy7. At the indicated time points (0 h, 30 min, 2 h, 6 h, 12 h, 24 h, and 48 h), mice were anesthetized with 2.5% isoflurane and then placed in the in vivo Imaging System (FXPro Kodak). The excitation and emission bandpass filter was 730 nm and 790 nm, respectively. Fluorescence signals were collected by Carestream Molecular Imaging Software. At 12 h, mice were euthanized and the ex vivo optical images of the intestine, heart, liver, spleen, lung, and kidney were recorded using the same system.

2.7. Statistical Analysis. Data are expressed as means ± SEM and were analyzed by one-way ANOVA using GLM procedures. Statistical differences among treatments were determined using the Student-Newman-Keuls Multiple Range Test (Prism software, version 5). A P value < 0.05 was considered significant.

3. Results

3.1. Effect of Sublancin on the Phagocytic Activity of Peritoneal Macrophages Ex Vivo. In our previous study, we found that sublancin could activate macrophage cell line RAW264.7 cells and peritoneal macrophages in vitro (unpublished data). Therefore, we further investigate the effect of oral administration of sublancin on the phagocytic activity of peritoneal macrophages ex vivo. As shown in Figure 1(a), the sublancin treatment of 1.0 mg/kg significantly augmented the phagocytic activity of peritoneal macrophages ex vivo ($P < 0.01$). These findings are consistent with the results of the phagocytic activity obtained by RAW264.7 cells and peritoneal macrophages in vitro.

3.2. Effect of Sublancin on the Gene Expression of Cytokines in Peritoneal Macrophages In Vivo. As indicated in Figures 1(b) and 1(c), oral administration of sublancin for 7 days significantly enhanced the gene expression levels of IL-1β and IL-6 in peritoneal macrophages ($P < 0.05$). Additionally, the gene expression level of TNF-α was increased significantly in 1.0 mg/kg sublancin treatment compared with control ($P < 0.05$) (Figure 1(d)). For the 2.5 mg/kg levamisole hydrochloride, the gene expression levels of all these three cytokines were also higher than that in the control group ($P < 0.05$).

3.3. Effect of Sublancin on the Body Weight in Cyclophosphamide-Treated Mice. In order to evaluate the immunomodulatory effects of sublancin, a cyclophosphamide-induced immunosuppressed mouse model was established in this study. The body weight of mice in each treatment is summarized in Figure 2. Prior to the cyclophosphamide injection (day 1), the body weight did not differ ($P > 0.05$) among the treatment groups. All mice that were injected with cyclophosphamide exhibited a significant decrease in body weight compared with the normal control mice ($P < 0.05$). Twenty-four hours after the last drug administration (day 11), body weights were still poorer in the model control than in the normal control. Treatment with all 3 sublancin levels resulted in similar body weight to levamisole hydrochloride.

3.4. Sublancin Activates the Phagocytic Activity of the Macrophage System. The carbon clearance test was performed to investigate the effect of sublancin on macrophage phagocytic activity. As shown in Figure 3(a), the carbon clearance index α in cyclophosphamide-treated mice was significantly lower than that in the normal control ($P < 0.01$). However, the inhibitory effect of cyclophosphamide was significantly alleviated by sublancin. Mice in the three sublancin level treatments (2.0, 4.0, and 8.0 mg/kg) had higher carbon clearance index α values than those in the model control, indicating that sublancin can enhance the phagocytic activity of the reticuloendothelial system.

3.5. Effect of Sublancin on the Cytokine mRNA Expression in Cyclophosphamide-Treated Mice. It was found that cyclophosphamide treatment reduced the IL-2, IL-4, and IL-6 mRNA expression levels significantly in the MC group, compared with the NC mice as shown in Figure 3 ($P < 0.001$). As compared to the MC group, mice treated with sublancin at doses of 4.0 mg/kg and 8.0 mg/kg showed a significant increase in the mRNA levels of IL-2, IL-4, and IL-6 ($P < 0.05$).

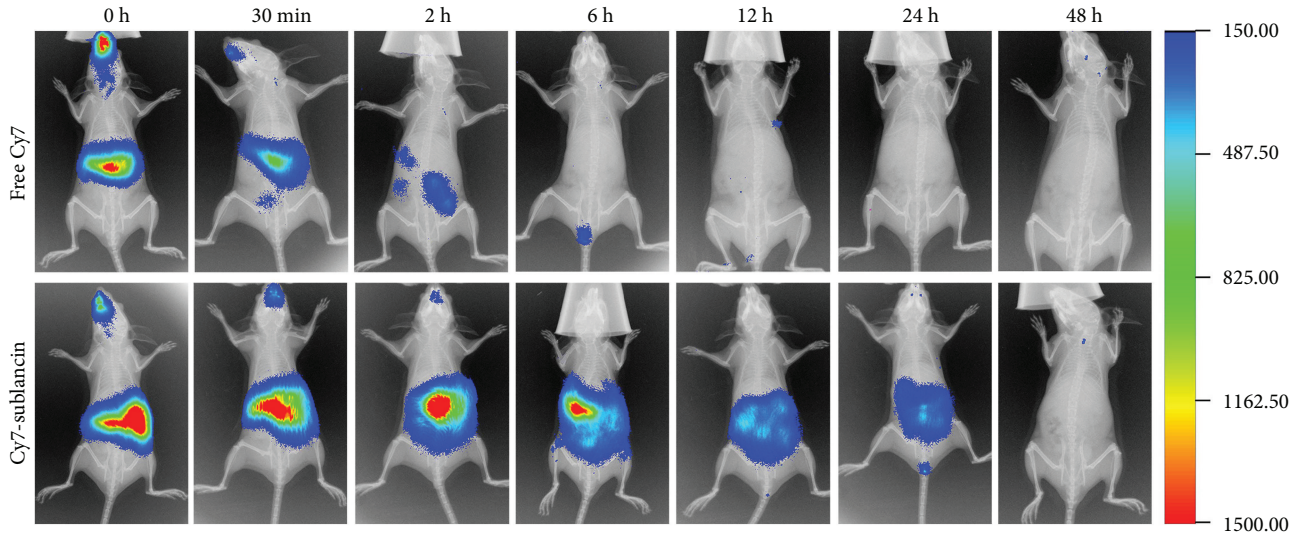

FIGURE 5: In vivo biodistribution of Cy7-labeled fluorescent sublancin in 6–8-week-old BALB/c mice evaluated by near-infrared fluorescent scans.

3.6. Effect of Sublancin on Hemopoietic Function in Cyclophosphamide-Treated Mice. In order to evaluate the protective effect of sublancin on the myelosuppression induced by sublancin, the RBC, WBC, HGB, and platelet numbers from peripheral blood were analyzed. As shown in Figure 4, peripheral RBC, WBC, HGB, and platelet counts were significantly decreased ($P < 0.05$) in the MC mice compared with the NC mice. Mice treated with 4.0 mg/kg and 8.0 mg/kg sublancin had higher ($P < 0.05$) RBC, HGB, and platelets than mice in the MC group. A numerical increase in the number of WBC was observed in the three sublancin treatments compared with the MC group. In addition, treatment with 10.0 mg/kg levamisole hydrochloride resulted in higher numbers of RBC, WBC, HGB, and platelets compared with the NC treatment. These findings indicated that sublancin can ameliorate myelosuppression induced by cyclophosphamide.

3.7. Intestinal Absorption of Sublancin. The intestinal absorption of sublancin was evaluated using the Caco-2 transwell system. Table 1 summarizes the apical and basolateral concentrations of sublancin and the calculated P_{app} at different time points of the transport experiment. After 3 h of incubation, $23.59 \pm 1.96\,\mu M$ of sublancin was detected on the basolateral side, corresponding with a P_{app} of $9.58 \pm 0.58 \times 10^{-6}\,cm \cdot s^{-1}$. It has been reported that compounds with poor absorption have P_{app} values less than $10 \times 10^{-6}\,cm \cdot s^{-1}$ [18]. The results from the current study indicated that sublancin was poorly absorbed by the Caco-2 monolayer.

3.8. In Vivo Biodistribution of Sublancin. To investigate the in vivo biodistribution of sublancin, the sublancin labeled with Cy7 was given orally to 6–8-week-old female BALB/c mice. As shown in Figure 5, Cy7-stained sublancin was continuously retained in the mouse intestine 24 h after the oral administration. In contrast, mice gavaged with free Cy7 showed weak fluorescent signals within 6 h. Twelve hours after administration, most of the free Cy7 had disappeared from the intestine. In addition, 12 h after gavage, the mice were euthanized and the intestine, heart, liver, spleen, lung, and kidney were excised to obtain ex vivo fluorescent images. As shown in Figure 6(a), a large amount of fluorescent Cy7-stained sublancin was continuously retained in the intestine. However, only very little of fluorescent free Cy7 was still located in the intestine. Almost no fluorescent signal was detected in the heart, liver, spleen, lung, and kidney of mice gavaged with Cy7-stained sublancin or free Cy7.

4. Discussion

The immune system plays a crucial role in the treatment of many diseases during chemotherapeutic intervention [19]. Emerging evidence indicates that modulation of immune response provides distinct advantages over conventional therapies [20, 21]. Macrophages are professional phagocytes for host defense against infections. Activated macrophages exhibit the ability to enhance phagocytosis and cytokine production [22]. It has been reported that orally administered AMPs could confer protection by immune modulation. Oral Pep19-2.5 (a synthetic AMP) administration was demonstrated to improve the clinical symptoms in *Salmonella typhimurium*-infected mice due to its ability to modulate immunity [23]. The AMP cecropin AD protected weaned piglets against *Escherichia coli* infection via increasing immune status [24]. Here, we show that oral administration of sublancin to BALB/c mice for 7 days enhanced phagocytic activity of peritoneal macrophages and promoted the mRNA expression of IL-1β, IL-6, and TNF-α by peritoneal macrophages. Additionally, a lack of change in the total number of peritoneal cells or other immune cell subset was observed after

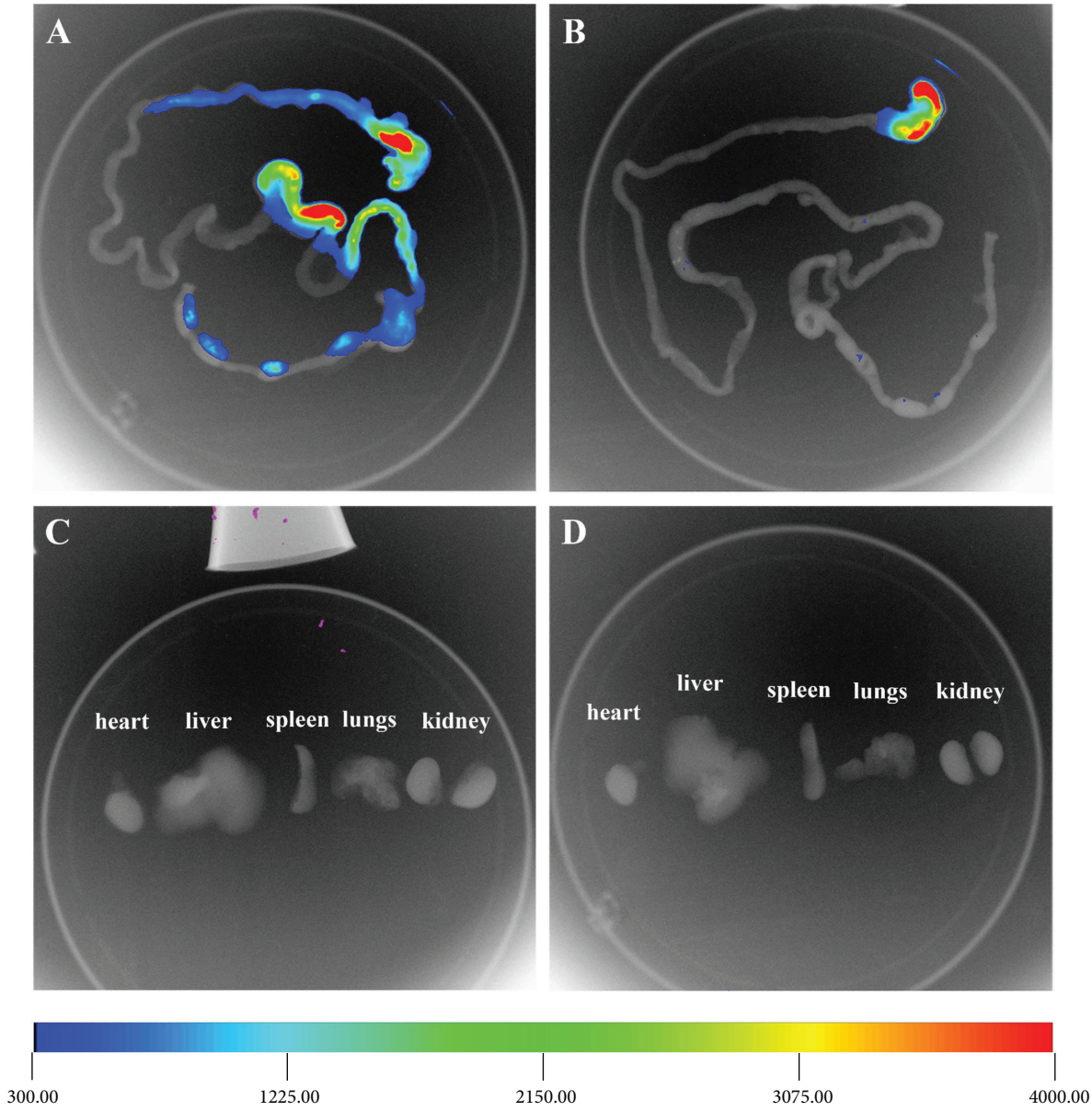

Figure 6: The ex vivo optical images of the intestines and organs of 6–8-week-old BALB/c mice with oral administration of Cy7-labeled fluorescent sublancin at 12 h. Monitor of the Cy7-labeled sublancin in the excised intestines (a) and organs (c); monitor of the free Cy7 in the excised intestines (b) and organs (d).

exposure of mice to various doses of sublancin (Figure S1). These observations suggest that sublancin could effectively activate macrophages and consequently improve the innate immunity.

It is well accepted that cyclophosphamide is an important chemotherapeutic drug in tumor treatment. However, cyclophosphamide also has adverse effects on healthy cells and results in immunosuppression [25]. In the present study, mice treated with cyclophosphamide were used as an immunosuppression model. Cyclophosphamide treatment resulted in a significant decrease in body weight. However, no difference was observed in the body weight among the sublancin-treated groups and the MC group on day 11, a result similar to the effects of levamisole hydrochloride.

Phagocytes (neutrophils, monocytes, and macrophages) are important actors in the innate immune response and are also the earliest cell types in response to pathogen invasions [26]. Macrophages are the most important phagocytes, and they play a pivotal role in host defense against diverse types of invasive cells, such as tumor cells [27]. Using immunomodulators for improving antitumor functions of macrophages is an area with good prospect for cancer chemotherapy [28]. The function of macrophages was evaluated with the carbon clearance test. Our results indicate that cyclophosphamide treatment impaired phagocytic activity of the macrophage system, which is consistent with previous reports [29, 30]. However, oral sublancin administration substantially enhanced the phagocytic

activity of the reticuloendothelial system in immunosuppressed mice, indicating that sublancin improved innate immune function of immunosuppressed mice.

Myelosuppression induced by cyclophosphamide administration is a major problem for cancer patients in clinical chemotherapy. In agreement with the results of previous studies [31, 32], cyclophosphamide treatment markedly decreased the count of peripheral RBC, WBC, HGB, and platelets. We observed that sublancin restored peripheral RBC, WBC, HGB, and platelet counts, suggesting that sublancin could protect against myelosuppression induced by cyclophosphamide.

T lymphocytes play an important role in recognizing and presenting antigens. IL-2 is a Th1-derived cytokine that is crucial for cell-mediated immune responses. IL-4 and IL-6 are produced by Th2 cells and can promote humoral immunity [33]. It was found that sublancin treatment had no significant effects on the total number of splenic cells and immune cell subset in the spleen (Figure S1). In the current study, it showed that the mRNA expression levels of IL-2, IL-4, and IL-6 in the spleen were significantly higher in the sublancin-treated groups (4.0 and 8.0 mg/kg) than in the cyclophosphamide-treated group, suggesting that sublancin could facilitate the recovery of the mRNA expression levels of IL-2, IL-4, and IL-6 in the spleen and alleviate the severity of immunosuppression.

To date, the mechanism of action of AMPs has been well studied and established, but most naturally occurring AMPs are abandoned during the development phase due to their potential lability to proteases or limited bioavailability [34]. The gastrointestinal absorption characteristic is a key issue in early drug development. At present, the half-life of AMPs in the gut after oral administration or whether they can be absorbed by the intestine is poorly reported [35]. Confluent Caco-2 monolayers, cultured on permeable supports, are commonly used to predict the intestinal absorption of drugs [36]. Compounds which are poorly absorbed in the human intestine had $P_{app} < 10 \times 10^{-6}$ cm·s^{-1} in the Caco-2 transwell system, and compounds with complete absorption had $P_{app} > 70 \times 10^{-6}$ cm·s^{-1} [18]. According to the abovementioned correlations, sublancin showed poor absorption with a P_{app} of $9.58 \pm 0.58 \times 10^{-6}$ cm·s^{-1} after 3 hours of incubation. In a previous study, the antimicrobial peptide sublancin did not show any signs of cytotoxicity in the Caco-2 cells as determined by the CCK-8 assay [8], indicating that the Caco-2 cell monolayer may not compromised by sublancin. In addition, we also determined the sublancin concentration in the cell lysate. No sublancin was detected in the cells (data not shown). Hence, we speculate that the observed poor flux might be caused by the paracellular transport. Next, we observed the in vivo biodistribution of Cy7-stained sublancin using the in vivo Imaging System. We found that Cy7-stained sublancin continuously retained in the intestine 24 h after oral administration, whereas mice orally administered with free Cy7 showed weak fluorescent signals within 6 h. This result indicated that Cy7-stained sublancin stayed longer in the intestine than free Cy7. Moreover, almost no fluorescent signal was observed in the heart, liver, spleen, lung, and kidney of mice gavaged with Cy7-stained sublancin 12 h after administration, suggesting that sublancin may not be absorbed into the circulation system by gavage within 12 h. It has been reported that sublancin has an extraordinary high chemical stability, tolerating both low and high pH, as well as gastrointestinal digestive enzymes [7, 37]. We speculate that sublancin and the fluorophore were excreted when the fluorescent signal was no longer detected in the intestine.

5. Conclusion

In conclusion, our study demonstrates the immunostimulatory properties of sublancin, which effectively activated macrophages and consequently improved the innate immunity of mice in vivo. Further, oral sublancin administration accelerated the recovery of immunosuppression in cyclophosphamide-treated mice. All these results indicate that sublancin is a potential candidate for use in immune therapy regimens.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

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

Acknowledgments

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Supplementary Materials

Figure S1: effects of sublancin on the immune cell subset in the peritoneal cavity (PerC) and spleen in vivo. BALB/c mice were orally administered with sublancin for 28 days (0.3 mg/kg and 1.0 mg/kg) or 14 days (0.6 mg/kg and 1.2 mg/kg), and the peritoneal cells and spleen were collected 24 hours after the last dose for flow cytometry. (A and B) A lack of change in the total number of peritoneal cells and splenic cells was observed after exposure of mice to various doses of sublancin. (C) Comparison of the mean percentage and cell numbers of peritoneal myeloid cells (CD 11b+), B cells (B220+), macrophages (F4/80+), and neutrophils (Ly6G+) in the peritoneal cavity between sublancin and control mice. (D) The average percentage and cell numbers of CD4+ T cells (CD4+), CD8+ T cells (CD8+), B cells (B220+), and NK cells (NK1.1+) in the spleen of sublancin and control mice are compared. Values are expressed as means ± SEM. *(Supplementary Materials)*

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Research Article

MicroRNA Expression Profiling in Behçet's Disease

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Background. Behçet's disease (BD) is a chronic inflammatory multisystem disease characterized by oral and genital ulcers, uveitis, and skin lesions. MicroRNAs (miRNAs) are key regulators of immune responses. Differential expression of miRNAs has been reported in several inflammatory autoimmune diseases; however, their role in BD is not fully elucidated. We aimed to identify miRNA expression signatures associated with BD and to investigate their potential implication in the disease pathogenesis. *Methods*. miRNA microarray analysis was performed in blood cells of BD patients and healthy controls. miRNA expression profiles were analyzed using Affymetrix arrays with a comprehensive coverage of miRNA sequences. Pathway analyses were performed, and the global miRNA profiling was combined with transcriptoma data in BD. Deregulation of selected miRNAs was validated by real-time PCR. *Results*. We identified specific miRNA signatures associated with BD patients with active disease. These miRNAs target pathways relevant in BD, such as TNF, IFN gamma, and VEGF-VEGFR signaling cascades. Network analysis revealed several miRNAs regulating highly connected genes within the BD transcriptoma. *Conclusions*. The combined analysis of deregulated miRNAs and BD transcriptome sheds light on some epigenetic aspects of BD identifying specific miRNAs, which may represent promising candidates as biomarkers and/or for the design of novel therapeutic strategies in BD.

1. Introduction

Behçet's disease (BD) is a rare and chronic multisystem disease characterized by a triple-symptom complex of recurrent oral aphthous ulcers, genital ulcers, and uveitis. Moreover, manifestations of vascular, articular, neurologic, urogenital, gastrointestinal, pulmonary, and cardiac involvement may occur. Hippocrates described BD in the fifth century BCE. In 1930, the Greek ophthalmologist Benediktos Adamantiades reported a patient with inflammatory arthritis, oral and genital ulcers, phlebitis, and iritis. The disease is named after the Turkish dermatologist Hulusi Behçet, who identified it in a patient in 1924 and published a description of the disease in 1937.

As virtually no unique histological or laboratory features have been identified to help in the diagnosis of the disease, clinical features are used to define and diagnose Behçet syndrome. An international study group on Behçet's disease has recently revised the criteria for the classification/diagnosis of BD [1].

There are sporadic cases of BD all around the world, but it is most frequently seen along the ancient Silk Route because of its frequency in the Middle East and far-east Asia (prevalence of 14–20/100,000 inhabitants), and these regions have traditionally been considered the endemic areas for the condition [2].

BD is a sporadic disease, but a familial aggregation is well known. Carriers of HLA-B51/HLA-B5 have an increased risk of developing Behçet's disease compared with noncarriers. HLA-B51 is the strongest associated genetic factor, and it has been shown to be more prevalent in Turkish, Middle Eastern, and Japanese populations, with a higher prevalence

of Behçet's disease in these populations [3]. Non-HLA genes also contribute to the development of BD [3]. Genome-wide association studies have shown that polymorphisms in genes encoding for cytokines, activator factors, and chemokines are associated with increased BD susceptibility. Among cytokines, IL-10 polymorphisms cause a reduction in the serum level of IL-10, an inhibitory cytokine that regulates innate and adaptive immune responses; on the other hand, IL-23 receptor polymorphism, which reduces the response to IL-23 stimulation, is associated with protection from BD [3–5]. Recent data reported also associations with CCR1, STAT4, and KLRC4 encoding for a chemokine receptor, a transcription factor implicated in IL-12 and IL-23 signaling and a natural killer receptor [6, 7]. Finally, susceptibility genes involved in the innate immune response to microbial exposure have recently been identified by Immunochip analysis [8].

Increased Th1, $CD4^+$ and $CD8^+$ T cell, $\gamma\delta^+$ T cell, and neutrophil activities have been found both in the serum and in inflamed tissues of BD patients, suggesting the involvement of innate and adaptive immunity in the pathogenesis of BD [2, 9]. Studies on T lymphocytes have suggested a Th1-predominant response. Both $CD4^+$ and $CD8^+$ lymphocytes are higher in the peripheral blood, with increased levels of IL-2 and interferon- (INF-) γ cytokines [10]. The cytokine Th17 may also play an important role in the pathogenesis of the disease [2, 11]. This hypothesis is supported by the observation of high IL-21 and IL-17 levels in sera of patients affected by BD with neurologic involvement [12, 13]. Another study has reported a higher Th17/Th1 ratio in peripheral blood of patients with BD compared to healthy controls, and this ratio was higher in patients with uveitis or folliculitis compared with patients without these manifestations [14, 15].

MicroRNAs (miRNAs) are small noncoding RNAs that play an important role in the regulation of several biological processes through their interaction with cellular messenger RNAs [16]. Inflammatory responses have an impact on miRNA expression, regulating their biogenesis by altering the transcription and processing of precursor transcripts or influencing the stabilization of mature miRNAs [17, 18]. In recent years, the number of miRNAs implicated in immune system development and function has dramatically enhanced, and there has been widespread discussion of their potential use as therapeutics for immunological diseases [16]. Indeed, the aberrant expression of miRNAs frequently occurs in human diseases, including hematological disorders and autoimmunity [19, 20]. The concept that miRNAs participate in the pathogenesis of diseases, especially refractory diseases with unidentified mechanisms, might lead to a novel effective treatment. A number of studies have reported a differential expression of miRNAs in several inflammatory autoimmune diseases, such as in rheumatoid arthritis (RA), multiple sclerosis, systemic lupus erythematosus, psoriasis, and systemic sclerosis [21]. These studies highlighted a deep implication of miRNAs as regulatory molecules in autoimmunity and the intriguing possibility to use miRNAs as disease biomarkers in these immunological disorders.

Table 1

Patients utilized for gene array study	6 (100%)
Sex	
Male	4
Female	2
Clinical features	
Aphthous stomatitis	6 (100%)
Genital ulcers	2 (33%)
Erythema nodosum-like lesions	1 (17%)
Papulopustular lesion	5 (83%)
Uveitis	2 (33%)
Epididymitis	0
Neurological symptoms	0
Vasculitis	4 (67%)
Joint manifestation	3 (50%)
Gastrointestinal involvement	0
Association with HLA-B51	4 (67%)

As far as BD concerns, little is known about miRNA expression; moreover, no high-throughput miRNA expression studies have been conducted to identify miRNAs specifically associated with the disease and no study has been so far performed which combines the analysis of blood microRNAs with transcriptional profiles in patients with BD.

In the present study, we performed a miRNA microarray analysis on peripheral blood mononuclear cells (PBMCs) of BD patients. We identified specific miRNA signatures associated with patients with active BD. These deregulated miRNAs target signaling pathways typically implicated in BD pathogenesis, such as TNF, interferon gamma, and VEGF and VEGFR signaling cascades.

The modular analysis of differentially expressed genes in BD revealed pathogenetically relevant networks that are possibly targeted by the identified miRNAs. This study sheds light on some aspects of BD pathogenesis identifying deregulated miRNAs as promising candidates for the discovery of disease biomarkers and/or as molecular tools for designing novel therapeutic strategies in BD.

2. Materials and Methods

2.1. Patients. A group of 6 subjects with BD was used for the gene array study. All the patients attended the Unit of Autoimmune Diseases at the University Hospital in Verona, Italy.

All patients fulfilled the International Criteria for Behçet Disease (ICBD): oral aphthosis, genital ulcers, and ocular lesions were each given 2 points, whereas 1 point was assigned to each of skin lesions, vascular manifestations, and neurological manifestations. A patient scoring 4 points or above was classified as having BD [22, 23]. At enrollment, none of the patients had active infections or was affected by malignancies.

The clinical features of the patients are reported in Table 1 that also includes a description of the BD patients selected for the gene array study.

A written informed consent was obtained from all the participants to the study. The study was approved by the local Ethical Committee of the Azienda Ospedaliera Universitaria of Verona, Verona, Italy. All investigations have been conducted according to the principles expressed in the Helsinki declaration.

2.2. Microarray Analysis. Blood samples were collected in BD Vacutainer K_2EDTA tubes using a 21-gauge needle. Peripheral blood mononuclear cells (PBMC) were obtained upon stratification on Lympholyte® cell separation density gradient (Cedarlane, Burlington, Canada). Total RNA extraction from PBMC was performed with miRNeasy Mini Kit following the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). RNA concentration and purity were evaluated by spectrophotometric analysis (NanoDrop 2000; Thermo Fisher Scientific, Wilmington, DE, USA), and a further check for RNA integrity was performed with 2100 Bioanalyzer (Agilent Genomics, Santa Clara, CA, USA) before microarray hybridization. Sample hybridization and scanning were performed as recommended by the Affymetrix (Affymetrix; Thermo Fisher Scientific Inc.) supplied protocols, by the Cogentech Affymetrix Microarray Unit (Campus IFOM-IEO, Milan, Italy), and Affymetrix GeneChip® miRNA 4.0 (Affymetrix; Thermo Fisher Scientific Inc., Waltham, MA, USA) was used. The GeneChip miRNA 4.0 arrays contain more than 30,000 probes including those encoding for 2578 mature human miRNAs, according to Sanger miRBase v.20.

The arrays were analyzed employing the Transcriptome Analysis Console (TAC) 4.0 software (Applied Biosystems, Foster City, CA USA, by Thermo Fisher Scientific, Waltham, MA, USA). The Signal Space Transformation- (SST-) Robust Multiarray Average (RMA) algorithm was applied to background-adjust, normalize, and log-transform signal intensity.

Relative expression levels of each microRNA were validated applying a one-way analysis of variance (ANOVA) and false discovery rate (FDR) correction ($p \leq 0.01$). MicroRNAs with an expression level of at least 1.5-fold different in the test sample versus control sample were analyzed.

Targeted genes of significantly modulated miRNAs were identified using the integrative database for human microRNA target predictions mirDIP [24]. All the source filters and a very high confidence class were applied for our analyses.

Pathway enrichment analysis of miRNA gene targets and differentially expressed genes (DEGs) in Behçet's disease was carried out using FunRich (http://www.funrich.org/) [25], and only Bonferroni-corrected enriched p values ≤0.01 calculated by the hypergeometric test were considered.

Pathways enrichment analysis of DEGs in BD was also performed employing the Panther expression analysis tools (http://pantherdb.org/) [26].

2.3. Protein-Protein Interaction (PPI) Network Construction and Network Clustering. Differentially expressed genes (DEGs) in BD samples from our previous analyses (Puccetti et al. unpublished observations) were mapped to the STRING database (version 1.0; http://string-db.org/) [27] to detect protein-protein interactions (PPI) pairs validated by experimental studies. A score of ≥0.7 for each PPI pair was selected to construct the PPI network. For the topological analysis of the built network, Cytoscape software was used and network clustering analysis was performed with the MCODE plugin of Cytoscape, based on the thresholds of module score > 1.5 [28].

2.4. Real-Time PCR. Mature miRNA expression was assayed by TaqMan® Advanced miRNA assay chemistry (Applied Biosystems, Foster City, CA, USA). Briefly, 10 ng of total RNA was reverse transcribed and preamplified with TaqMan Advanced miRNA cDNA synthesis kit following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Preamplified cDNA was diluted 1/10 in nuclease-free water, and 5 μL of diluted cDNA for each replicate was loaded in PCR. 20 μL PCR reactions were composed by 2x Fast Advanced Master Mix and TaqMan Advanced miRNA assays for hsa-miR-143-3p (477912_mir), hsa-miR-199a-5p (478231_mir), and hsa-miR-4505 (477842_mir). The mean of Ct for hsa-miR-16-5p (477860_mir) and hsa-miR-26a-5p (477995_mir) expression was used to normalize miRNA expression. Real-time PCR was carried out in triplicate on a QuantStudio 6 Flex instrument (Applied Biosystems, Foster City, CA, USA). Expression values were reported as fold change with respect to healthy controls by the ΔΔCt method using QuantStudio Real-Time PCR system software versus 1.3.

3. Results

3.1. High-Throughput MicroRNA Expression Profiling in Peripheral Blood Mononuclear Cells of Behçet's Disease. Since a global miRNA expression analysis in BD with an updated coverage of miRNA sequences has not been performed yet, we wanted to provide a careful description of miRNAs associated with BD by interrogating the transcription of a large amount of different microRNA sequences in BD PBMCs by microarray strategy.

Therefore, PBMCs derived from 6 patients with BD and 6 healthy age- and sex-matched donors were analyzed using a dedicated and high-density array with a coverage for more than 2500 human microRNA transcripts and all mature miRNA sequences in miRBase Release 20. The clinical features of patients included in the microarray study are reported in Table 1.

Microarray analysis revealed a high number (269) of modulated miRNAs that satisfied the FDR-corrected p value criterion ($p \leq 0.01$) and the fold change criterion (FC ≥ |1.5|), showing a robust and statistically significant variation between BD and healthy control samples (Supplementary Table 1). Such a large number of modulated transcripts clearly reflect the high performance of the array in the detection of a wide range of microRNA sequences. We thereafter sharpened our analysis by selecting only modulated miRNAs annotated as "high confidence" in miRBase 21 (http://www.mirbase.org), and to make our results more informative, we further narrowed down the analysis to miRNAs for which gene targets were annotated in FunRich.

By these criteria, we selected 47 modulated miRNAs that are shown in Table 2. Interestingly, almost all (45/47) miRNAs were down-modulated with only two up-regulated microRNAs.

Selected miRNAs significantly deregulated in the microarray analysis were validated by real-time PCR in the entire series of patients analyzed (see Figure 1).

3.2. Pathway Enrichment Analysis of miRNAs Deregulated in Behçet's Disease. In a second part of our analysis, we wanted to identify all the molecular pathways that were targeted by the selected miRNA performing a pathway enrichment analysis based on annotated gene targets in FunRich. The software allows to evaluate the miRNA regulatory effect and to identify controlled pathways based on predicted and/or validated miRNA-target interactions. We also applied a pathway enrichment analysis on the dataset of differentially expressed genes (DEGs) obtained in our previous study of gene expression profiling in BD (Puccetti et al., unpublished observations). In this study, we were able to select modulated genes that may play an important role in BD pathogenesis since they are involved in biological processes strongly connected to the typical features of the disease.

Despite the strong statistical stringency applied to the two datasets ($p \leq 0.01$), we obtained a high number of significantly enriched pathways both from the miRNA target datasets (277) and from the BD DEGs (164) (Supplementary Tables 2 and 3). Notably, we found that a large proportion of pathways from the BD DEGs was also enriched in the list of miRNA-validated targets (64%, 106/164; Supplementary Table 4), indicating that the selected miRNAs exert a strong impact on the molecular pathways altered in the disease. Moreover, the large number of enriched pathways clearly reflected the multisystemic involvement typical of Behçet's disease. Figure 2 shows a graphical representation of selected commonly enriched pathways. Interestingly, the enriched categories were involved in vascular biology (i.e., glypican pathway, vascular endothelial growth factor, VEGF and VEGFR network, endothelin pathway, PAR1-mediated thrombin signaling events, thrombin/protease-activated receptor (PAR) pathway, EGFR-dependent endothelin signaling events, platelet-derived growth factor (PDGF) receptor signaling network, and urokinase-type plasminogen activator (uPA), and uPAR-mediated signaling) and in apoptosis (TRAIL, p53, and FAS signaling pathways). In addition, other relevant pathways enriched in the two datasets were related to the immune response (i.e., IL6-mediated signaling events, TCR and BCR signaling, calcineurin-regulated NFAT-dependent transcription in lymphocytes, and toll-like receptor cascades) and to the inflammatory response (i.e., tumour necrosis factor, TNF receptor, IFN-gamma, p38 MAP kinase, pathway, and IL1- and CXCR4-mediated signaling events.

3.3. Comparative Analysis of Selected miRNA Gene Targets and Differentially Expressed Genes in BD. To better define the role played by miRNAs in BD pathogenesis, we wanted to select miRNAs that were able to target genes modulated in BD. Therefore, we used a more sophisticated integrative database for human microRNA target predictions (mirDIP) [24] to obtain the lists of genes that were targeted, with a very high score class, by each of the selected miRNAs. Then, we compared the resulting target lists to genes differentially expressed in BD from our previous study (Puccetti et al., unpublished observations) and we observed that 65% of DEGs were targeted by the selected miRNAs. Interestingly, the vast majority of these DEGs showed an opposite modulation with respect to the relative targeting miRNA (Figure 3(a)), consistently with the typical role of miRNAs as negative regulator of gene expression (i.e., typically, up-regulated genes are targeted by down-modulated miRNAs and underexpressed genes are targeted by overexpressed miRNAs). All the above-mentioned miRNAs are presented in Figure 3(b) along with the compiled lists of their targets DEGs.

All these selected miRNAs targeted genes involved in biological processes implicated in the disease pathogenesis including apoptosis, immune response, inflammation, and vascular damage (Figure 4). Thus, we could identify microRNAs that may control the gene modulation involved in the disease pathogenesis. Table 3 shows all the targeted genes and their corresponding targeting miRNAs.

Selected miRNAs targeted many DEGs that sustained the inflammatory response typically associated with BD including TNF, IL1A, IL10, IL6R, CXCL2, CXCR4, TNFAIP3, OLR1, S100A8, HSP90B1, and CCL3 (see Figure 3(b) and Table 3). Interestingly, TNF (targeted by hsa-miR-181d-5p and -181a-5p), CCL3 (targeted by hsa-let-7e-5p, -7d-5p, and -7f-5p), IL10 (targeted by hsa-let-7f-5p, -7e-5p, -7d-5p, -miR-146a-5p, and -27b-3p), and IL1A (targeted by hsa-miR-505-3p, -181d-5p, and -181a-5p) have been detected at increased concentrations in sera or plasma of BD patients when compared to normal subjects [29–31]. Moreover, down-modulated miRNAs targeted DEGs involved in the adaptive immune response including genes that played a role in T and in B cell immune response. Among these genes, several miRNAs targeted CD28 (hsa-miR-143-3p, -27b-3p, -15b-5p, and -195-5p), CD4 (hsa-miR-181a-5p), ICOS (hsa-miR 27b-3p and -let-7f-5p, -7e-5p, and -7d-5p), CTLA4 (hsa-miR-143-3p), EGR1 (hsa-miR-143-3p, -146a-5p, -181a-5p, -199b-3p, and -199a-3p), and AKIRIN2 (hsa-miR-139-5p, -27b-3p, and -181a-5p). Interestingly, 18 miRNAs targeted genes of the TH-17 gene signature including CD28, CD4, ICOS, SOCS3, IL6ST, YY1, IL6R, TNF, CXCL2, and SOCS1 (Figure 3(b)).

In addition, selected miRNAs could also control DEGs involved in the innate immune response including, for example, NKTR (hsa-miR-181a-5p, -486-5p, -151-3p, and -27b-3p), KIR2DL4 (hsa-miR-146a-5p), STAT1 (hsa-miR-584-5p, -27b-3p, -128-3p, and -146a-5p), STAT2 (hsa-miR-143-3p), and DEGs belonging to the Toll-like receptor (TLR) pathway such as TLR2, IKBKB, JUN, REL, NAMPT, HSP90B1, HSPA4, and ARF3. In particular, we have to mention that TLR2 (targeted by hsa-miR-143-3p and -146a-5p) is thought to be up-regulated in BD patients [32, 33]. As many as 22 miRNAs targeted genes of the type I interferon signature like VEGFA, DDX3X, and the above-mentioned STAT1, STAT2, SOCS1, and IL10. The coexpression of miRNAs

Table 2: miRNAs modulated in BD patients versus healthy subjects.

ID	Probe set name	Transcript ID (array design)	Fold change	FDR p value	Accession
20518901	MIMAT0019041_st	hsa-miR-4505	9.46	0.0001	MIMAT0019041
20500781	MIMAT0004609_st	hsa-miR-149-3p	6.05	0.0003	MIMAT0004609
20500119	MIMAT0000065_st	hsa-let-7d-5p	−1.55	0.0066	MIMAT0000065
20500444	MIMAT0000256_st	hsa-miR-181a-5p	−1.76	0.0095	MIMAT0000256
20500778	MIMAT0000449_st	hsa-miR-146a-5p	−1.87	0.0032	MIMAT0000449
20501197	MIMAT0000703_st	hsa-miR-361-5p	−1.88	0.0013	MIMAT0000703
20503908	MIMAT0004780_st	hsa-miR-532-3p	−1.92	0.0032	MIMAT0004780
20502123	MIMAT0004748_st	hsa-miR-423-5p	−2	0.0052	MIMAT0004748
20501036	MIMAT0000617_st	hsa-miR-200c-3p	−2.12	0.0032	MIMAT0000617
20501182	MIMAT0000692_st	hsa-miR-30e-5p	−2.17	0.0081	MIMAT0000692
20500158	MIMAT0000085_st	hsa-miR-28-5p	−2.2	0.0016	MIMAT0000085
20500422	MIMAT0000244_st	hsa-miR-30c-5p	−2.49	0.0019	MIMAT0000244
20501276	MIMAT0000751_st	hsa-miR-330-3p	−2.53	0.0089	MIMAT0000751
20500797	MIMAT0000460_st	hsa-miR-194-5p	−2.6	0.0028	MIMAT0000460
20502124	MIMAT0001340_st	hsa-miR-423-3p	−2.61	0.0025	MIMAT0001340
20500159	MIMAT0004502_st	hsa-miR-28-3p	−2.66	0.0012	MIMAT0004502
20500718	MIMAT0000417_st	hsa-miR-15b-5p	−2.94	0.0005	MIMAT0000417
20500424	MIMAT0000245_st	hsa-miR-30d-5p	−2.99	0.0003	MIMAT0000245
20500795	MIMAT0004614_st	hsa-miR-193a-5p	−3.01	0.0015	MIMAT0004614
20500385	MIMAT0000222_st	hsa-miR-192-5p	−3.06	0.0019	MIMAT0000222
20500758	MIMAT0000438_st	hsa-miR-152-3p	−3.06	0.0046	MIMAT0000438
20500151	MIMAT0000081_st	hsa-miR-25-3p	−3.2	0.0003	MIMAT0000081
20503811	MIMAT0002821_st	hsa-miR-181d-5p	−3.3	0.0005	MIMAT0002821
20500123	MIMAT0000067_st	hsa-let-7f-5p	−3.34	0.0041	MIMAT0000067
20504274	MIMAT0003218_st	hsa-miR-92b-3p	−3.43	0.0018	MIMAT0003218
20500162	MIMAT0000087_st	hsa-miR-30a-5p	−3.74	0.0006	MIMAT0000087
20500488	MIMAT0000280_st	hsa-miR-223-3p	−4.32	0.0043	MIMAT0000280
20503887	MIMAT0002876_st	hsa-miR-505-3p	−4.58	0.0023	MIMAT0002876
20500733	MIMAT0000424_st	hsa-miR-128-3p	−5.07	0.0029	MIMAT0000424
20501291	MIMAT0000759_st	hsa-miR-148b-3p	−5.43	0.0084	MIMAT0000759
20501278	MIMAT0000752_st	hsa-miR-328-3p	−5.63	0.0029	MIMAT0000752
20500798	MIMAT0000461_st	hsa-miR-195-5p	−5.8	0.0012	MIMAT0000461
20500121	MIMAT0000066_st	hsa-let-7e-5p	−5.98	0.0052	MIMAT0000066
20500187	MIMAT0004514_st	hsa-miR-29b-1-5p	−6.81	0.0093	MIMAT0004514
20504378	MIMAT0003297_st	hsa-miR-628-3p	−6.83	0.0032	MIMAT0003297
20500170	MIMAT0004507_st	hsa-miR-92a-1-5p	−6.84	0.0005	MIMAT0004507
20500723	MIMAT0000419_st	hsa-miR-27b-3p	−7.22	0.0041	MIMAT0000419
20504553	MIMAT0004819_st	hsa-miR-671-3p	−8.16	0.0006	MIMAT0004819
20501287	MIMAT0000757_st	hsa-miR-151a-3p	−8.47	0.0084	MIMAT0000757
20503105	MIMAT0002177_st	hsa-miR-486-5p	−17.98	0.0066	MIMAT0002177
20500400	MIMAT0000232_st	hsa-miR-199a-3p	−21.75	0.0049	MIMAT0000232
20500458	MIMAT0004563_st	hsa-miR-199b-3p	−21.75	0.0049	MIMAT0004563
20500769	MIMAT0000445_st	hsa-miR-126-3p	−26.32	0.0023	MIMAT0000445
20504312	MIMAT0003249_st	hsa-miR-584-5p	−51.18	0.002	MIMAT0003249
20500399	MIMAT0000231_st	hsa-miR-199a-5p	−63.55	0.0023	MIMAT0000231
20500432	MIMAT0000250_st	hsa-miR-139-5p	−66.67	0.0027	MIMAT0000250
20500752	MIMAT0000435_st	hsa-miR-143-3p	−83.47	0.0046	MIMAT0000435

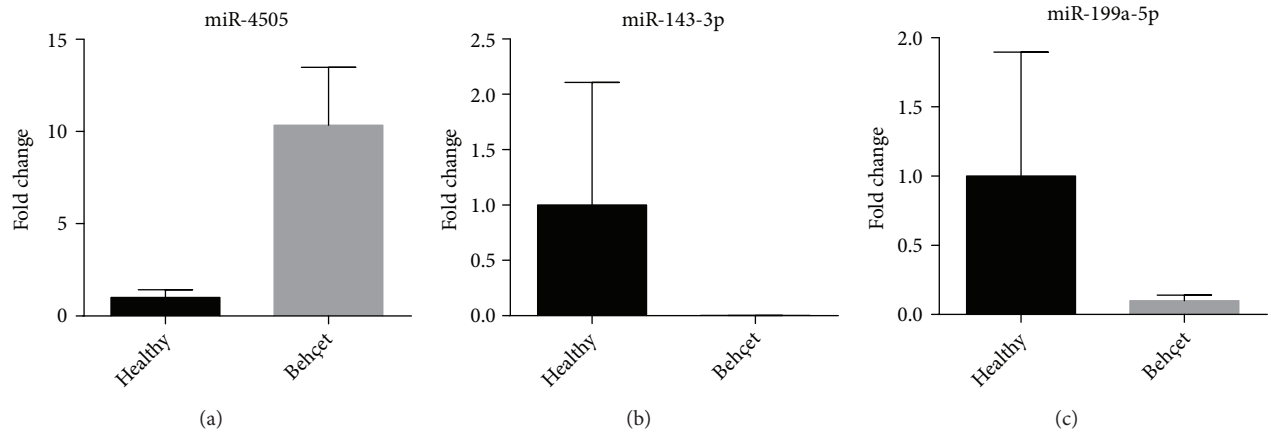


Figure 1: Validation of differentially expressed miRNAs by real-time PCR. Real-time PCR for the indicated miRNAs were performed in healthy controls (Healthy) and in BD patients (Behçet). Values are calculated as fold change with respect to healthy samples with the ΔΔCt method. miR-16-5p and miR-26-5p were used as endogenous controls for miRNA expression (see Material and Methods). Histograms indicate mean values; bars indicate standard deviation (SD). $p = 0.05$ (Mann–Whitney test).

targeting DEGs involved in the type I IFN signaling and Th-17 related genes may reflect the presence of a synergy between IFN and Th17 pathways that is typical of autoimmune diseases [34–43], thus suggesting that an autoimmune mechanism can be involved in the BD pathogenesis.

Noteworthily, several miRNAs targeted all the eight genes involved in the JAK/STAT signaling pathway that we found up-regulated in our previous work (Puccetti et al., unpublished observations) including PIK3R1 and the above-mentioned STAT1, STAT2, IL6ST, IL6R, IL10, SOCS1, and SOCS3. The downmodulation of miRNAs targeting this pathway further supports the hypothesis of an autoimmune origin of BD since its activation is very frequently associated with autoimmune diseases [44]. Moreover, the JAK/STAT signaling pathway is active in $CD4^+$ T cells of patients with BD [45].

Interestingly, modulated miRNAs targeted DEGs involved in the vascular damage associated with BD that is characterized by myointimal proliferation, fibrosis, and thrombus formation [46]. Indeed, several miRNAs targeted DEGs associated with blood coagulation (i.e., THBS1, F5, and LMAN1; see Table 3), a process whose alteration is typically associated with BD vasculitis. Noteworthily, 10 down-modulated miRNAs (hsa-let-7d/7e/7f-5p, hsa-miR-151-3p, -628-3p, -139-5p, 143-3p, -194-5p, -199a-3p, and -199b-3p) targeted THBS1, suggesting a crucial role of THBS1 in the pathogenesis of vascular damage. Other modulated miRNAs targeted DEGs that played a role in angiogenesis including MMP8, VEGFA, NR4A3, and NAPA. Moreover, NR4A3 is a transcription factor involved in vascular development [47] and NAPA promotes vascular endothelial- (VE-) cadherin localization at endothelial junctions [48]. Interestingly, increased serum levels of soluble VE-cadherins have been detected in BD patients [49]. Among the above-mentioned genes, we have to notice that VEGFA was predictively targeted by a high number (9) of down-modulated miRNAs, including hsa-miR-199a-3p, -15b-5p, -195-5p, -361-5p, -126-3p, -486-5p, -199a-5p, 199b-3p, and hsa-miR-139-5p. Other DEGs involved in vascular damage and targeted by modulated miRNAs were FOSL2, THBD, and PTX3. THBD, targeted by hsa-miR-139-5p, is increased in sera of BD patients compared with healthy subjects, and PTX3, targeted by hsa-miR-628-3p, is considered as a marker of small vessel vasculitis [50].

Moreover, DEGs involved in the apoptotic process were targeted, and among these, we can cite MCL1, DNAJB1, BCL2L11, ARHGDIA, ZNF331, and IER3 (Table 3). Finally, several modulated miRNAs targeted DEGs that played a role in cell proliferation, a process that can be induced in response to both apoptosis and to skin ulcer formation. Among these miRNAs, we can mention those that targeted BTG2, EREG, PRRC2, and APLP2. In particular, EREG (targeted by hsa-miR-199a-3p, -199b-3p, and -181a-5p and by hsa-miR-192-5p) and APLP2 (targeted by hsa-miR-199a-3p, -199b-3p, -139-5p, and -181a-5p) were involved in the proliferation of corneal epithelial cells and in corneal epithelial wound healing, respectively [51, 52]. Thus, this gene regulation may have a role in ocular manifestations of BD like keratitis.

3.4. Network Analysis of Genes Differentially Expressed in BD. We performed a network analysis in which the functional interactions between the protein products of modulated genes in BD were evaluated. By this approach, a protein-protein interaction (PPI) network comprising 171 genes (nodes) and 3272 pairs of interactions (edges) was constructed (Figure 5(a)). We then performed a clustering analysis to identify areas of densely interconnected nodes (clusters/modules; CL) that are predicted to be involved in common biological processes and to have a crucial role in the disease pathogenesis. We could detect six clusters that are graphically represented in Figures 5(b)–5(g). Comparing the list of miRNA targets to DEGs included in the six clusters (Supplementary Table 5), we found that, in each cluster, a large number of DEGs were targeted (Supplementary Table 6 and Figures 5(b)–5(g)). In particular, we observed that several of these genes were involved in immune and inflammatory responses including TNF (CL2), IL10 (CL2), and IL1A (CL1). Moreover, many of such genes played a

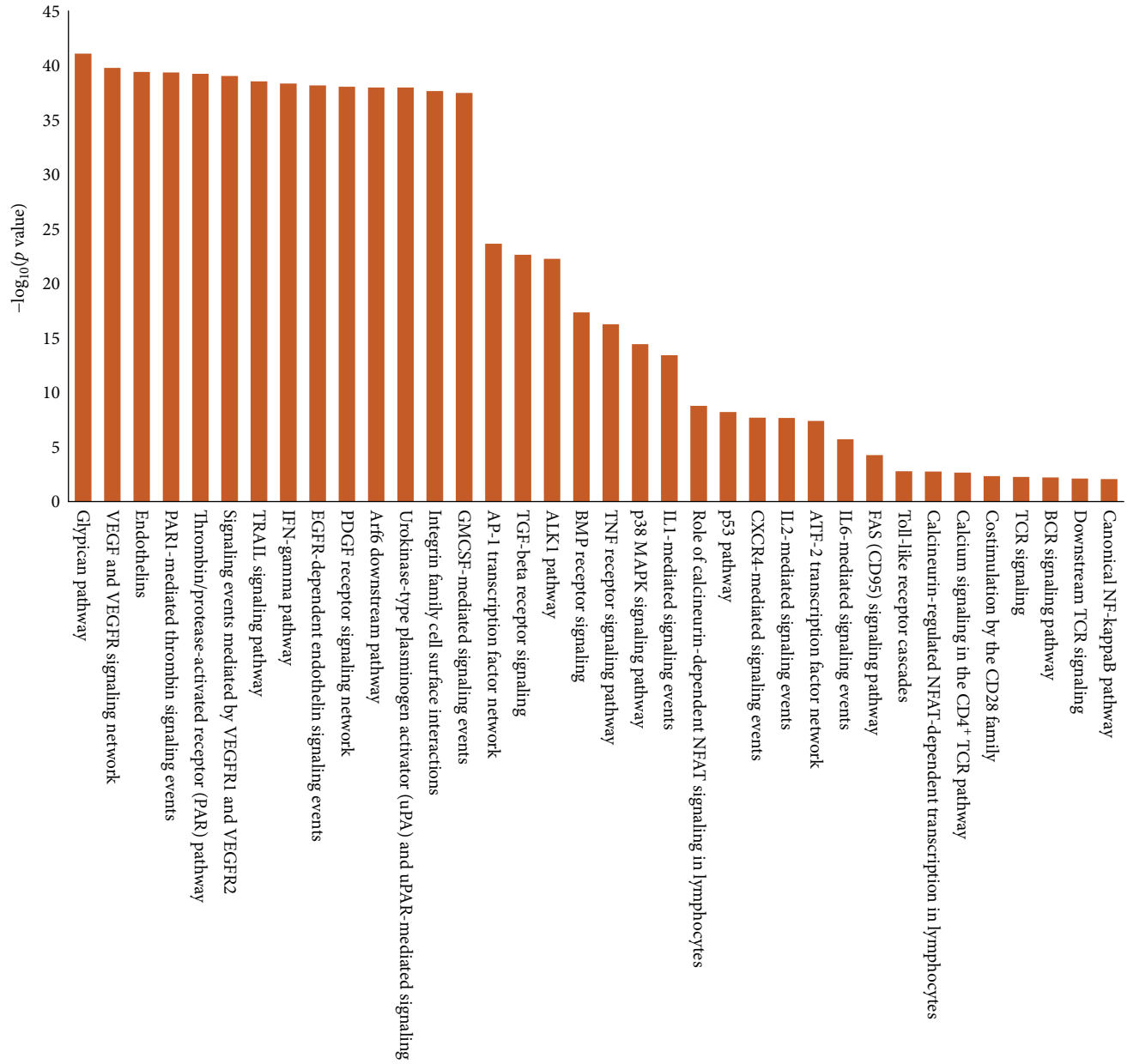
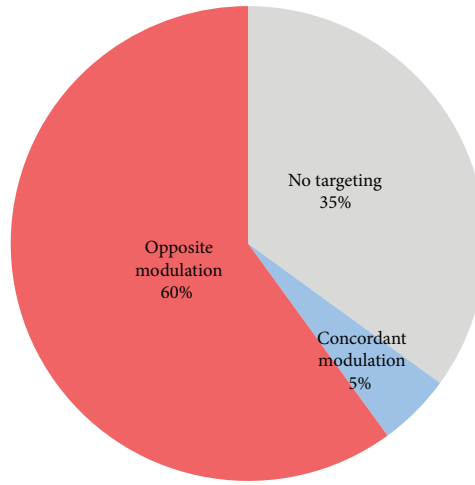


Figure 2: Histogram representing pathways enriched in BD-modulated miRNA targets genes and in BD DEGs. y axis: $-\log_{10}(p \text{ value})$ (hypergeometric test).

role in B cell response, like EGR1 (CL1), or in T cell response like, for example, CTLA4, CD4, and MLL (CL1); DUSP2 (CL3); and CD28 (CL4) and NR4A2 (CL5). Other DEGs targeted in clusters were involved in TLR signaling including HSPA4, IKBKB, JUN, REL, S100A8, TLR2 (CL1), and ARF3 and NAMPT (CL2). Interestingly, in the six clusters, we also observed that various genes involved in angiogenesis and/or in vascular damage were targeted by selected miRNAs including THBS1, MMP8, VEGFA (CL1), FOSL2, THBD (CL2), HIPK1 and DDX6 (CL3), PGK1 (CL4), and ACTR2 (CL6).

Given the well-known biological significance of highly connected gene clusters, to gain insight on the most relevant signaling networks that were controlled by the deregulated miRNAs, we performed a pathway enrichment analysis on targeted genes that were present in the six clusters. All the above-mentioned enriched pathways are listed in the Supplementary Table 7, and Table 4 shows a selection of the most relevant enriched signaling networks.

The TLR and the JAK/STAT signaling networks, two pathways notoriously involved in immune response and frequently associated with autoimmunity, were enriched in CL1-targeted genes (CL1-TGs). Other pathways implicated in the immune response were enriched in several genes targeted in clusters including T cell activation (CL1-TGs, CL2-TGs, and CL4-TGs), B cell activation (CL1-TGs), and PI3K signaling (CL2-TGs and CL3-TGs). This pathway is a crucial element in the regulation of adaptive and innate immune response [53, 54] and is a key player in inflammatory response. Therefore, in recent



(a)

hsa-miR-126-3p	hsa-miR-139-5p	hsa-miR-143-3p	hsa-miR-151-3p	hsa-miR-199a-3p	hsa-miR-199a-5p	hsa-miR-199b-3p	hsa-miR-27b-3p	hsa-miR-29b-1-5p	hsa-miR-486-5p	hsa-miR-584-5p	hsa-miR-628-3p	hsa-miR-671-3p	hsa-let-7d-5p	hsa-miR-361-5p	hsa-miR-423-5p	hsa-miR-200c-3p	hsa-miR-30e-5p	hsa-miR-30c-5p
PLK2 PIK3R1 VEGFA	CXCR4 CALM1 DDX3X JUN AKIRIN2 AP2M1 TCF3 CREM DAZAP2 KAT6A MCL1 RBL2 APLP2 TGIF1 THBD THBS1 VEGFA MMP8 DDX6 HIPK1	CXCR4 CALM1 TSC22D3 HSPD1 **IL6R** DUSP1 MARCKS **CD28** CD84 CTLA4 EGR1 **IL6ST** STAT2 TLR2 KAT6A PPP2R1A MLL SIKE1 DUSP2 FLOT1 FOSL2 AP2M1 DNAJB1 PGK1 DDX6 HIPK1 THBS1	CALM1 IKZF3 NKTR DAZAP2 PTPN12 MCL1 THBS1 ACTR2	CALM1 DUSP1 KMT2A NAMPT PDE4B PIK3R1 SIKE1 EGR1 BCL2L11 MCL1 EREG RBL2 APLP2 HIPK1 ACTR2 TGIF1 THBS1 VEGFA	MARCKS CREM DDX3X PPP2R1A STK4 IKBKB IKZF1 HSPA4 BTG2 DDX6 HIPK1 VEGFA	CALM1 KMT2A MCL1 MLL NAMPT PDE4B PIK3R1 EGR1 SIKE1 BCL2L11 EREG APLP2 ACTR2 VEGFA TGIF1 THBS1 HIPK1	TNFAIP3 PLK2 MAP4K5 MARCKS HSPD1 AKIRIN2 ARF3 IKZF1 **IL10** **IL6ST** **CD28** DAZAP2 DDX3X MLL NKTR PIK3R1 PTGER4 STAT1 **ICOS** KAT6A KMT2A ZFP36L2 DNAJB1 IER3 PRRC2C BTG2 PGK1 TGIF1	DAZAP2 GNAI2	NKTR PIK3R1 PTPN12 STK4 ZNF331 DNAJB1 PRRC2C VEGFA HIPK1	MARCKS STAT1 MCL1	HSPD1 PTX3 STK4 MCL1 RBL2 THBS1	DDX3X	BTG2 CALM1 CCL3 DUSP1 **ICOS** **IL10** OLR1 S100A8 **SOCS1** THBS1	NR4A2 VEGFA	AP2M1	DGKA DUSP1 FSTL1 IKBKB JUN MARCKS	BCL2L11 FOSL2 GNAI2 NR4A2 **SOCS1** **SOCS3**	BCL2L11 FOSL2 GNAI2 NR4A2 **SOCS1** **SOCS3**

hsa-miR-330-3p	hsa-miR-194-5p	hsa-miR-15b-5p	hsa-miR-30d-5p	hsa-miR-192-5p	hsa-miR-152-3p	hsa-miR-25-3p	hsa-miR-181d-5p	hsa-let-7f-5p	hsa-miR-92b-3p	hsa-miR-30a-5p	hsa-miR-223-3p	hsa-miR-505-3p	hsa-miR-128-3p	hsa-miR-148b-3p	hsa-miR-195-5p	hsa-let-7e-5p	hsa-miR-181a-5p	hsa-miR-146a-5p
NAPA	PTPN12 THBS1	ACTR2 ARHGDIA BTG2 **CD28** IKBKB PIK3R1 PPP2R1A VEGFA	BCL2L11 FOSL2 GNAI2 NR4A2 **SOCS1** **SOCS3**	EREG	DDX6 DUSP1 HSP90B1 **IL6ST** LMAN1 PDE4B SNX27 **SOCS3**	BTG2 DUSP1 FOSL2 NR4A3 PTGER4 TGIF1	BCL2L11 CALM1 HSP90B1 IL1A **TNF**	BTG2 CALM1 CCL3 DUSP1 **ICOS** IL10 S100A8 **SOCS1** THBS1 **YY1**	BCL2L11 BTG2 FOSL2 NR4A3 PTGER4 TGIF1	BCL2L11 FOSL2 GNAI2 NR4A2 **SOCS1** **SOCS3**	HSP90B1 **IL6ST** INPP4A NLRP3 RHOB	HSPD1 IL1A **IL6ST**	MAP4K5 MARCKS PIK3R1 PLK2 STAT1 ZFP36L2	BCL2L11 DDX6 DUSP1 FLOT2 HSP90B1 **IL6ST** KMT2A LMAN1 PDE4B RAB35 SNX27 **SOCS3**	ACTR2 ARHGDIA BTG2 CALM1 **CD28** DDX3X FOSL2 IKBKB KMT2A PHACTR4 PIK3R1 PPP2R1A PRRC2C RAB35 TCF3 TSC22D3 VEGFA	BTG2 CALM1 CCL3 DUSP1 **ICOS** **IL10** **IL6R** OLR1 REL S100A8 **SOCS1** THBS1 TNFAIP3 **YY1**	ACTR2 AKIRIN2 AP1LP2 ARF3 BCL2L11 BTG2 CALM1 **CD4** **CXCL2** DAZAP2 DDX3X DNAJB1 EGR1 ERAP1 EREG F5 FSTL1 HIPK1 HSP90B1 IL1A **IL6ST** INPP4A KMT2A LMAN1 MARCKS MCL1 NKTR NR4A2 NR4A3 PHACTR4 PRRC2C **SOCS3** **TNF** **YY1** ZFP36L2	BTG2 DAZAP2 DDX3X EGR1 FLOT2 HIPK1 IKZF3 **IL10** KIR2DL4 KMT2A PGK1 PLK2 PTGER4 REL SIKE1 STAT1 TGIF1 TLR2 ZNF331

(b)

Figure 3: Selection of miRNAs that targeted genes differentially expressed in BD. (a) Pie chart showing the percentage of BD DEGs that were targeted by miRNAs modulated in BD. (b) BD-modulated miRNAs and their respective targeted BD DEGs. Genes associated with Th17 cells are written in bold characters.

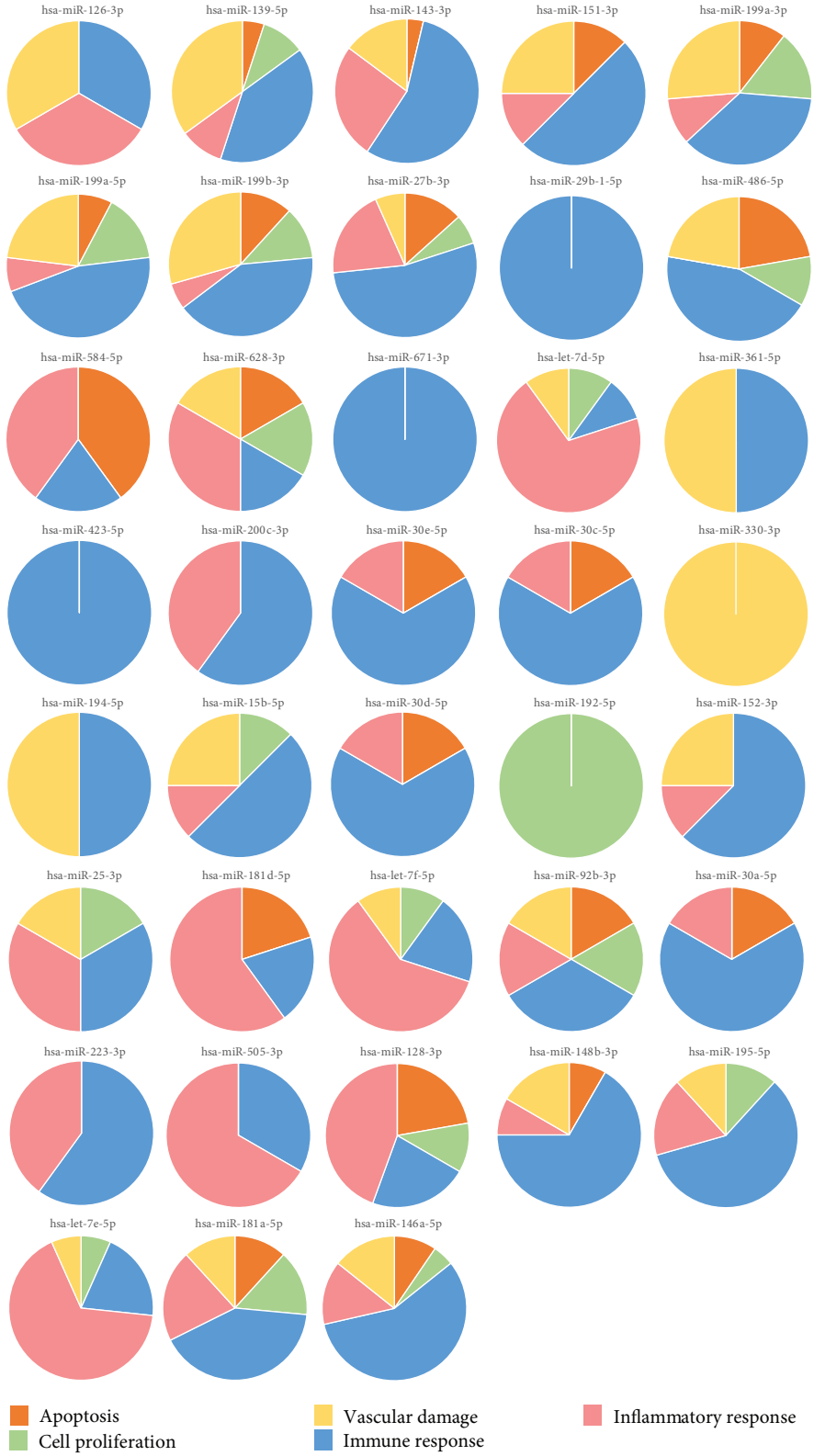


Figure 4: Functional classification of BD DEGs targeted by selected miRNAs. Pie charts showing the different GO biological processes in which BD DEGs targeted by selected deregulated miRNAs in BD can be classified.

years, highly selective inhibitors of PI3K have been developed for anti-inflammatory treatments [55]. An enrichment in signalings involved in the inflammatory response was also found in CL1-TGs (inflammation mediated by chemokine and cytokine and interleukin pathways) and in CL5-TGs (integrin pathway).

Table 3: List of all BD DEGs that were targeted by selected miRNAs modulated in BD and their respective targeting miRNAs.

DEGs	MicroRNA										
ACTR2	miR-181a-5p	miR-151-3p	miR-199a-3p	miR-199b-3p	miR-15b-5p	miR-195-5p					
AKIRIN2	miR-181a-5p	miR-27b-3p	miR-139-5p								
APLP2	miR-181a-5p	miR-139-5p	miR-199a-3p	miR-199b-3p							
ARF3	miR-181a-5p	miR-27b-3p									
BCL2L11	miR-181a-5p	miR-199a-3p	miR-199b-3p	miR-30e-5p	miR-30c-5p	miR-30d-5p	miR-181d-5p	miR-92b-3p	miR-30a-5p	miR-148b-3p	
BTG2	miR-181a-5p	miR-27b-3p	miR-199a-5p	let-7d-5p	miR-15b-5p	miR-25-3p	let-7f-5p	miR-92b-3p	miR-195-5p	let-7e-5p	miR-146a-5p
CALM1	miR-181a-5p	miR-139-5p	miR-143-3p	miR-151-3p	miR-199a-3p	miR-199b-3p	let-7d-5p	miR-181d-5p	let-7f-5p	miR-195-5p	let-7e-5p
CD4	miR-181a-5p										
CXCL2	miR-181a-5p										
DAZAP2	miR-181a-5p	miR-27b-3p	miR-139-5p	miR-151-3p	miR-29b-1-5p	miR-146a-5p					
DDX3X	miR-181a-5p	miR-27b-3p	miR-139-5p	miR-199a-5p	miR-671-3p	miR-195-5p	miR-146a-5p				
DNAJB1	miR-181a-5p	miR-27b-3p	miR-143-3p	miR-486-5p							
EGR1	miR-181a-5p	miR-143-3p	miR-199a-3p	miR-199b-3p	miR-146a-5p						
ERAP1	miR-181a-5p										
EREG	miR-181a-5p	miR-199a-3p	miR-199b-3p	miR-192-5p							
F5	miR-181a-5p										
HIPK1	miR-181a-5p	miR-139-5p	miR-143-3p	miR-199a-3p	miR-199a-5p	miR-199b-3p	miR-486-5p	miR-146a-5p			
HSP90B1	miR-181a-5p	miR-152-3p	miR-181d-5p	miR-223-3p	miR-148b-3p						
IL1A	miR-181a-5p	miR-181d-5p	miR-505-3p								
IL6ST	miR-181a-5p	miR-27b-3p	miR-143-3p	miR-152-3p	miR-223-3p	miR-505-3p	miR-148b-3p				
INPP4A	miR-181a-5p	miR-223-3p									
KMT2A	miR-181a-5p	miR-27b-3p	miR-199a-3p	miR-199b-3p	miR-148b-3p	miR-195-5p	miR-146a-5p				
LMAN1	miR-181a-5p	miR-152-3p	miR-148b-3p								
MARCKS	miR-181a-5p	miR-27b-3p	miR-143-3p	miR-199a-5p	miR-584-5p	miR-200c-3p	miR-128-3p				
MCL1	miR-181a-5p	miR-139-5p	miR-151-3p	miR-199a-3p	miR-199b-3p	miR-486-5p	miR-584-5p	miR-628-3p			
NKTR	miR-181a-5p	miR-27b-3p	miR-151-3p								
NR4A2	miR-181a-5p	miR-361-5p	miR-30e-5p	miR-30c-5p	miR-30d-5p	miR-30a-5p					
NR4A3	miR-181a-5p	miR-25-3p	miR-92b-3p								
PRRC2C	miR-181a-5p	miR-27b-3p	miR-486-5p	miR-195-5p							
SOCS3	miR-181a-5p	miR-30e-5p	miR-30c-5p	miR-30d-5p	miR-152-3p	miR-30a-5p	miR-148b-3p				
TNF	miR-181a-5p	miR-181d-5p									
YY1	miR-181a-5p	let-7f-5p	let-7e-5p								
ZFP36L2	miR-181a-5p	miR-27b-3p	miR-128-3p								
TNFAIP3	miR-27b-3p	let-7e-5p									
PLK2	miR-27b-3p	miR-126-3p	miR-128-3p	miR-146a-5p							

Table 3: Continued.

DEGs	MicroRNA									
MAP4K5	miR-27b-3p	miR-128-3p								
HSPD1	miR-27b-3p	miR-143-3p	miR-628-3p	miR-505-3p						
IKZF1	miR-27b-3p	miR-199a-5p								
IL10	miR-27b-3p	let-7d-5p	let-7f-5p	let-7e-5p	miR-146a-5p					
CD28	miR-27b-3p	miR-143-3p	miR-15b-5p	miR-195-5p						
MLL	miR-27b-3p	miR-143-3p	miR-199a-3p	miR-199b-3p						
PIK3R1	miR-27b-3p	miR-126-3p	miR-199a-3p	miR-199b-3p	miR-486-5p	miR-15b-5p	miR-128-3p	miR-195-5p		
PTGER4	miR-27b-3p	miR-25-3p	miR-92b-3p	miR-146a-5p						
STAT1	miR-27b-3p	miR-584-5p	miR-128-3p	miR-146a-5p						
ICOS	miR-27b-3p	let-7d-5p	let-7f-5p	let-7e-5p						
KAT6A	miR-27b-3p	miR-139-5p	miR-143-3p							
VEGFA	miR-126-3p	miR-139-5p	miR-199a-3p	miR-199a-5p	miR-199b-3p	miR-486-5p	miR-361-5p	miR-15b-5p	miR-195-5p	
CXCR4	miR-139-5p	miR-143-3p								
JUN	miR-139-5p	miR-200c-3p								
AP2M1	miR-139-5p	miR-143-3p	miR-423-5p							
TCF3	miR-139-5p	miR-195-5p								
CREM	miR-139-5p	miR-199a-5p								
RBL2	miR-139-5p	miR-199a-3p	miR-628-3p							
TGIF1	miR-139-5p	miR-27b-3p	miR-199a-3p	miR-199b-3p	miR-25-3p	miR-92b-3p	miR-146a-5p			
THBD	miR-139-5p									
THBS1	miR-139-5p	miR-143-3p	miR-151-3p	miR-199a-3p	miR-199b-3p	miR-628-3p	let-7d-5p	miR-194-5p	let-7f-5p	let-7e-5p
MMP8	miR-139-5p									
DDX6	miR-139-5p	miR-143-3p	miR-199a-5p	miR-152-3p	miR-148b-3p					
PGK1	miR-27b-3p	miR-143-3p	miR-146a-5p							
TSC22D3	miR-143-3p	miR-195-5p								
IL6R	miR-143-3p	let-7e-5p								
DUSP1	miR-143-3p	miR-199a-3p	let-7d-5p	miR-200c-3p	miR-152-3p	miR-25-3p	let-7f-5p	miR-148b-3p	let-7e-5p	
CD84	miR-143-3p									
CTLA4	miR-143-3p									
STAT2	miR-143-3p									
TLR2	miR-143-3p	miR-146a-5p								
PPP2R1A	miR-143-3p	miR-199a-5p	miR-15b-5p	miR-195-5p						
SIKE1	miR-143-3p	miR-199b-3p	miR-199a-3p	miR-146a-5p						
DUSP2	miR-143-3p									
FLOT1	miR-143-3p									

Table 3: Continued.

DEGs	MicroRNA							
FOSL2	miR-143-3p	miR-30e-5p	miR-30c-5p	miR-30d-5p	miR-25-3p	miR-92b-3p	miR-30a-5p	miR-195-5p
IKZF3	miR-151-3p	miR-146a-5p						
PTPN12	miR-151-3p	miR-486-5p	miR-194-5p					
NAMPT	miR-199a-3p	miR-199b-3p						
PDE4B	miR-199a-3p	miR-199b-3p	miR-152-3p	miR-148b-3p				
STK4	miR-199a-5p	miR-486-5p						
IKBKB	miR-199a-5p	miR-200c-3p	miR-15b-5p	miR-195-5p				
HSPA4	miR-199a-5p							
GNAI2	miR-29b-1-5p	miR-30e-5p	miR-30c-5p	miR-30d-5p	miR-30a-5p			
ZNF331	miR-486-5p	miR-146a-5p						
PTX3	miR-628-3p							
CCL3	let-7d-5p	let-7f-5p	let-7e-5p					
OLR1	let-7d-5p	let-7e-5p						
S100A8	let-7d-5p	let-7f-5p	let-7e-5p					
SOCS1	let-7d-5p	miR-30e-5p	miR-30c-5p	miR-30d-5p	let-7f-5p	miR-30a-5p	let-7e-5p	
DGKA	miR-200c-3p							
NAPA	miR-330-3p							
ARHGDIA	miR-15b-5p	miR-195-5p						
SNX27	miR-152-3p	miR-148b-3p						
NLRP3	miR-223-3p							
RHOB	miR-223-3p							
FLOT2	miR-148b-3p	miR-146a-5p						
RAB35	miR-148b-3p	miR-195-5p						
REL	let-7e-5p	miR-146a-5p						
KIR2DL4	miR-146a-5p							

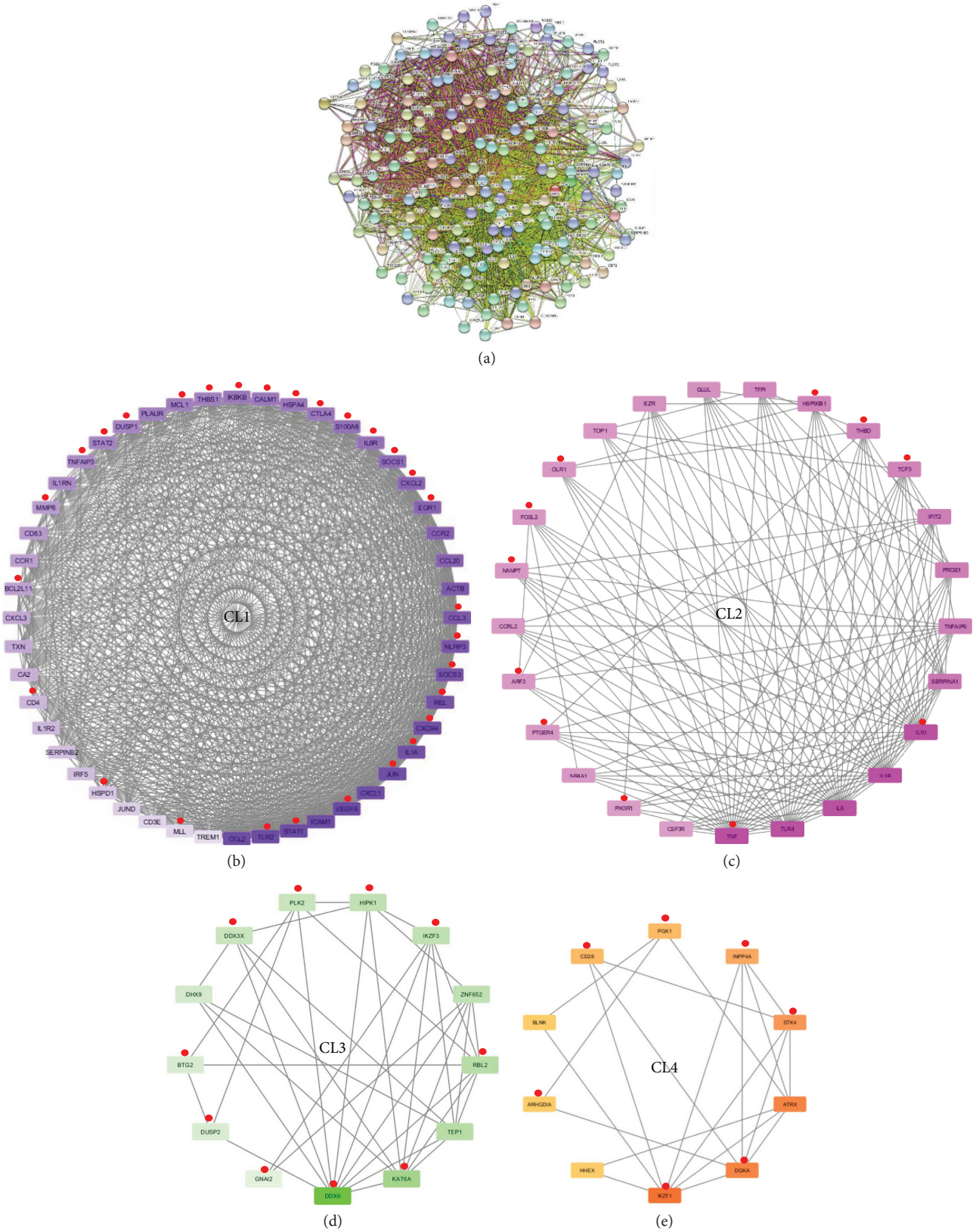


Figure 5: Continued.

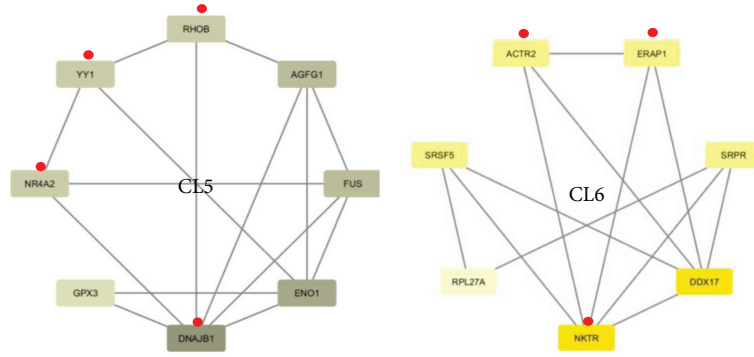


Figure 5: Network analysis of genes modulated in Behçet's disease. (a) PPI network of differentially expressed genes in BD. (b–g) Graphical representation of the six clusters that were extracted from the PPI network of modulated genes in BD. Red dots indicate DEGs that are targeted by modulated miRNAs in BD.

In CL1-TGs and in CL3-TGs, the oxidative stress response pathway was enriched. Interestingly, an increased oxidative stress has been described in BD and it has been correlated to the severe inflammatory and degenerative clinical manifestations of the disease [56]. Several networks clearly connected to vascular damage were enriched in cluster TGs including PDGF, angiogenesis (CL1-TGs and CL5-TGs), Wnt, blood coagulation, endothelin, VEGF (CL2-TGs), and cadherin (CL6-TGs) signaling pathway. VE-cadherins have a crucial role in endothelial barrier integrity, and noteworthily, in CL6 the ACTR2 gene was targeted, known to stabilize adherens junctions between endothelial cells of the vascular wall interacting with cadherins [57]. Moreover, the Wnt signaling pathway is critically involved in vascular biology [58]. Finally, in CL1-TGs and in CL2-TGs the apoptosis and the p53 pathways were enriched, respectively.

4. Discussion

A systematic analysis of miRNA expression profiles in BD has not been performed yet. The aim of our work is therefore to provide a compiled description of miRNAs associated with BD using an array able to query a very large number of transcripts in the attempt to dissect the possible regulatory effects exerted by these molecules on the molecular pathways relevant for the disease pathogenesis.

In a previous work, we investigated BD-associated transcriptional profiles by a gene expression analysis of PBMC derived from BD patients and identified a gene modulation strictly connected to the disease pathogenesis (Puccetti et al., unpublished observations). Moreover, we showed the presence of a type I interferon and Th-17 gene signature, which suggests an autoimmune origin of BD. In this study, we aimed to complement this gene expression analysis detecting modulated miRNAs that may target differentially expressed genes (DEGs) identified in our previous analysis.

To this purpose, we used a sophisticated target prediction system (mirDIP) [24] to obtain the list of miRNA targets, which was then compared to the list of DEGs identified in blood samples of BD patients. The comparison of the two analyses (gene expression and miRNAs) allows selecting within deregulated miRNAs only those related to the modulation of genes that would be effectively altered in the PBMCs of BD patients and might have therefore a pivotal role in the pathogenesis of the disease.

We observed that there was a good overlap (60%) between the selected miRNA targets and the BD-modulated genes, indicating that the majority of the identified miRNAs regulate genes differentially expressed in BD. Such target genes belonged to functional classes strictly connected to typical features of BD. Indeed, several miRNAs targeted pro-inflammatory genes such as TNF and IL1A. Moreover, transcripts involved in both adaptive and innate immune response were also targeted. In this regard, we have to mention that selected down-modulated miRNAs controlled several Th17 cell-associated genes and transcripts involved in type I interferon response that we found up-regulated in our previous analysis. This may suggest a loss of control in BD on two synergistic mechanisms typically associated with an autoimmune response. We moreover observed a down-regulation of miRNAs that control members of TLRs and JAK/STAT pathways, two molecular signalings involved in autoimmune diseases [44, 59] that are also active in BD [32, 60, 61]. In addition, miRNAs target genes involved in angiogenesis, and in blood coagulation, two processes commonly associated with BD vasculitis were also down-modulated in BD samples.

Consistently with the tight correspondence between deregulated miRNA and DEGS in BD, we observed a good overlap (64%) between the pathways enriched in the deregulated miRNA targets and in the genes differently expressed in BD. This indicated that, globally, the pathways identified by the selected miRNAs reflected fairly well the gene regulation described in our gene expression study, suggesting that the criteria applied for the selection of miRNAs had effectively identified miRNAs able to explain the gene modulation which we had previously described.

Interestingly, we found that both in BD-miRNA and in BD-DEGs dataset, meaningful signaling networks including,

Table 4: Pathways enriched in DEGs targeted in each cluster.

Panther pathways	p value
CL1	
Toll-like receptor signaling pathway	$8.44E-07$
Apoptosis signaling pathway	$2.34E-05$
Inflammation-mediated by chemokine and cytokine signaling pathway	$3.09E-05$
Interleukin signaling pathway	$1.68E-04$
PDGF signaling pathway	$1.14E-03$
Oxidative stress response	$1.46E-03$
B cell activation	$1.80E-03$
T cell activation	$3.49E-03$
JAK/STAT signaling pathway	$3.81E-03$
Angiogenesis	$1.79E-02$
CL2	
Wnt signaling pathway	$1.20E-02$
Hypoxia response via HIF activation	$1.87E-02$
Blood coagulation	$2.03E-02$
Insulin/IGF pathway-protein kinase B signaling cascade	$2.31E-02$
p53 pathway feedback loops 2	$2.87E-02$
PI3 kinase pathway	$3.15E-02$
VEGF signaling pathway	$3.64E-02$
Endothelin signaling pathway	$4.41E-02$
T cell activation	$4.74E-02$
p53 pathway	$4.90E-02$
CL3	
5HT1-type receptor-mediated signaling pathway	$2.07E-02$
PI3 kinase pathway	$2.63E-02$
Oxidative stress response	$2.72E-02$
CL4	
Glycolysis	$6.63E-03$
T cell activation	$2.79E-02$
CL5	
PDGF signaling pathway	$2.71E-02$
Angiogenesis	$3.03E-02$
Integrin signaling pathway	$3.04E-02$
CL6	
Cadherin signaling pathway	$2.10E-02$

for example, VEGF and VEGFR, endothelins, PDGF receptor, TNF receptor, and IL1- and IL6-mediated TCR and BCR pathways were enriched.

A pivotal task in molecular biology is to understand gene modulation in the context of biological networks. Indeed, proteins achieve their functions in the protein-protein-interacting network, and interestingly, the dynamics of such networks can be influenced by miRNAs [62]. We therefore wanted to identify significant relationships among modulated miRNAs and the PPI network in which the protein product of modulated genes in BD can be involved. Moreover, since it is known that the repressive effect of a miRNA may lead to more severe biological effects when it is exerted on proteins with more interacting partners [62], to highlight more efficient interrelations, we focused our attention on the most connected proteins of the network, inspecting the presence of miRNA targets inside the six clusters extracted from the PPI network. We found that the six clusters identified were extensively targeted by several modulated miRNAs, thus indicating that the deregulation of selected miRNAs may have a meaningful effect on the dynamics of a protein network that control the disease pathogenesis. Indeed, genes targeted in the six clusters played an important role in vascular biology (i.e., VEGFA, THBS, THBS1, etc.), in inflammation (i.e., TNF, IL1A, IL6R, CXCR4, etc.), and in immune response (i.e., CD28, CTLA4, EGR1, TLR2, etc.). Moreover, the analysis of pathways that were enriched in the target genes present in the clusters confirmed the essential role of these transcripts and their relative targeting miRNA in BD pathogenesis.

In conclusion, this work represents the first analysis performed on such a large number of miRNAs and integrated with the study of the profiles of gene expression in BD. The study allowed correlating the expression of miRNAs and the modulation of genes important for the pathogenesis of the disease. Using this approach, we have been able to identify the specific molecular pathways on which the regulation of these miRNAs may occur.

This study sheds light on some epigenetic aspects of BD identifying specific miRNAs, which may represent promising candidates for the identification of disease biomarkers and/or the design of novel therapeutic strategies in BD.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Antonio Puccetti, Claudio Lunardi, and Marzia Dolcino conceived and designed the experiments. Piera Filomena Fiore and Andrea Pelosi performed the experiments. Marzia Dolcino and Andrea Pelosi analyzed the data. Giuseppe Patuzzo selected the patients and contributed reagents. Marzia Dolcino wrote the paper with inputs from Claudio Lunardi and Antonio Puccetti. Antonio Puccetti, Andrea Pelosi, Claudio Lunardi, and Marzia Dolcino equally contributed to this paper.

Supplementary Materials

Supplementary 1. Supplementary Table 1: miRNAs significantly modulated in BD.

Supplementary 2. Supplementary Table 2: pathways enriched in genes targeted by modulated miRNAs in BD.

Supplementary 3. Supplementary Table 3: pathways enriched in differentially expressed genes in BD.

Supplementary 4. Supplementary Table 4: pathways enriched both in genes targeted by BD deregulated miRNAs and in BD differentially expressed genes.

Supplementary 5. Supplementary Table 5: differentially expressed genes in BD that were included in the six clusters.

Supplementary 6. Supplementary Table 6: differentially expressed genes in BD that were included in clusters that are targeted by modulated miRNAs in BD.

Supplementary 7. Supplementary Table 7: enriched pathways in target genes that are included in clusters.

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Review Article

Viral Modulation of TLRs and Cytokines and the Related Immunotherapies for HPV-Associated Cancers

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The modulation of the host innate immune system is a well-established carcinogenesis feature of several tumors, including human papillomavirus- (HPV-) related cancers. This virus is able to interrupt the initial events of the immune response, including the expression of Toll-like receptors (TLRs), cytokines, and inflammation. Both TLRs and cytokines play a central role in HPV recognition, cell maturation and differentiation as well as immune signalling. Therefore, the imbalance of this sensitive control of the immune response is a key factor for developing immunotherapies, which strengthen the host immune system to accomplish an efficient defence against HPV and HPV-infected cells. Based on this, the review is aimed at exposing the HPV immune evasion mechanisms involving TLRs and cytokines and at discussing existing and potential immunotherapeutic TLR- and cytokine-related tools.

1. Introduction

The evasion of immune tumor surveillance is a well-established feature of cancer [1]. In HPV-related tumors, papillomavirus is responsible for this escape (Figure 1). This virus is able to abrogate the initial steps of the immune innate system, which embraces Toll-like receptor signalling as well as cytokine synthesis and secretion, thus, compromising the immune response against an invasive agent [2].

TLRs and cytokines play pivotal roles in the immune defence against HPV-infected and tumor cells. TLRs, for example, are responsible for recognizing the conserved pathogen-associated molecular patterns (PAMPs), promoting changes on host endogenous ligands and thus, initiating a protein cascade that follows in the expression of key molecules for the development of the immune response, which includes the synthesis and secretion of cytokines [3]. Cytokines, in turn, are important mediators of immune cell activities, such as cell recruitment, maturation, and signalling [4]. In addition, both molecules (TLRs and cytokines) control gene expression and are essential for creating a suitable tumor microenvironment, either for immune surveillance or for immune modulation. As a result, these molecules are involved in the pathogenesis of various diseases besides cancer, such as autoimmune, inflammatory, and infectious diseases [5].

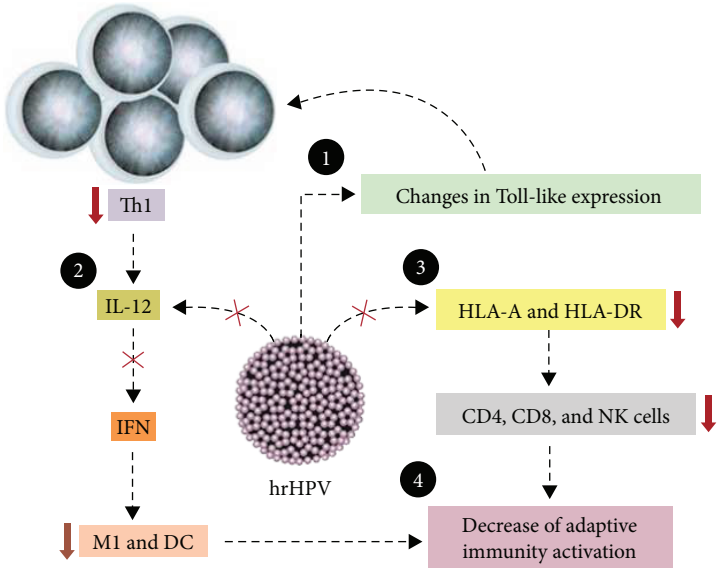

Figure 1: Th1 immune evasion induced by HPV in tumor microenvironment. HPV (1) interferes in TLR expression and in immune signalling pathways; (2) suppresses IL-12 expression, leading to a decreased production of IFNs (type I and IFN-γ) and blocking macrophages (M1 phenotype) and dendritic cell (DC) activity; (3) promotes downregulation of HLA expression and transportation to membrane surface, preventing antigen presentation, T cell activation, and NK and CTL cytotoxicity; and (4) finally, the downregulation of lymphocyte activity related to a decreased activity of APC cells (as M1 and DC) impairs adaptive immune activation [2].

Therefore, both TLRs and cytokines are crucial targets for immunotherapeutic studies that are aimed at preventing or treating cancer. In fact, they have already been used in pharmaceutical formulations for cancer therapies by taking advantage of the fact that currently there is no effective treatment for HPV-related cancer patients, especially for those who have unsuccessfully undergone radio- and chemotherapy treatments. Several major studies have reported the great potential value of immunotherapeutic approaches that modulated TLR and cytokine levels or synthesis [6, 7]. In accordance with these studies, this review highlights the immune mechanisms of TLRs and cytokines for infection resolution and viral immune evasion activities correlated with HPV-associated cancers. Furthermore, we will discuss the effectiveness of the immunotherapeutic approaches involving these targets.

2. Toll-Like Receptors

TLRs are specialized receptors which detect PAMPs and damage- or danger-associated molecular patterns (DAMPs). They are found in immune (e.g., APCs, natural killer) and nonimmune (e.g., stromal, epithelial, and cancer) cells on the plasma membrane (e.g., TLRs 1, 2, 4, 5, 6, and 10) and on the surface of organelles, such as endosomes, lysosomes, and endoplasmic reticulum (e.g., TLRs 3, 7, 8, and 9) [5].

The molecular structure of these receptors is comprised of two domains: the extracellular N-terminal and the intracellular C-terminal domains. The extracellular domain contains leucine-rich repeats responsible for recognition of PAMPs, depending on the TLR subtype; the C-terminal portion has a conserved region called Toll/IL-1 receptor (TIR) domain [5], which is responsible for transducing the signal for adapter molecules.

Usually, TLRs are associated with the protection against pathogen invasion, carcinogenesis, and infection clearance, which are essential in inducing and linking innate and adaptive responses, such as the Th1 and the cytotoxic cell-mediated subtypes [3]. In addition, TLRs are able to recognize some host endogenous ligands (see Figure 2), representing an important role in tissue repair and homeostasis [9].

TLRs support the uptake, processing, and presentation of antigens by APCs, boost DC maturation, NK cell cytotoxicity, and targeted cell apoptosis as well as upregulate the expression of major histocompatibility complex (MHC), C-C chemokine receptor 7 (CCR7), interferons (e.g., IFN-I, IFN-γ), and inflammatory cytokines (e.g., IL-6, IL-12) [3]. Nevertheless, several TLRs were reported to be overexpressed in cancer. They were associated with malignant transformation by preventing the activation of immune responses or enhancing inflammation through the induction of the nuclear transcription factor kappa B (NF-κB) pathway. Therefore, TLRs seem to have dual functions in the tumor microenvironment, to the extent that even cancer cells may express those molecules in order to alter immune response and sustain malignant progression [3, 10]. Indeed, recently, TLRs were showed to activate the nitric oxide signalling pathway, supporting cervical carcinogenesis [11].

The expression of both TLR4 and 9 receptors was reported to be altered during HPV infection. In cervical carcinogenesis studies, TLR4 and 9 levels were reported to be crucial for the initiation of the innate immune response due to the induction of cytokine synthesis and cytotoxicity on target cells. As a consequence, lower levels are generally associated with a poor prognosis and cancer progression [8, 12, 13]. However, these receptors have also been correlated with malignancy [3, 11, 14, 15]. TLR4 was found to be overexpressed in human cervical cancer line (HeLa) [11, 14] as well

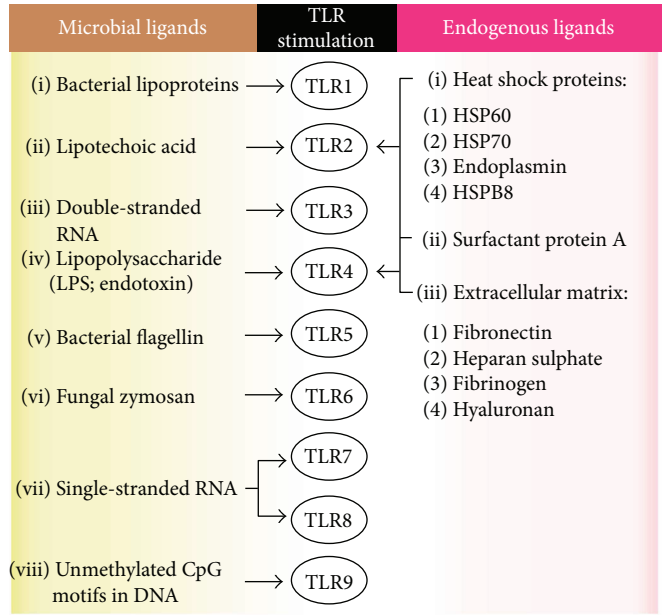

Figure 2: TLR activation. The scheme shows major microbial and endogenous ligands that activate TLR signals in immune cell surface. These signals are able to promote host protection against pathogen invasion and infection establishment [5].

as in premalignant and malignant specimens [16]. It was also associated with the proliferation of cancer cells and immunosuppression, through the production of IL-6, TGF-β, and other immunomodulatory cytokines [3, 10]. TLR9 was also shown to be overexpressed in high-grade cervical lesions and/or cancer [3, 13, 15–17]. It is possible that the upregulation of these receptors in malignant lesions may be due to the following components: (i) compensation for TLR deficiency or by harnessing mechanisms of the host immune defence system against tumor cells, (ii) reflecting increased inflammation, which damages an effective immune response against the pathogen, (iii) the existence of polymorphisms and the measurement of different subtypes, which misleads data interpretation, (iv) the measurements at different intervals during cancer progression, (v) the differences in methods of TLR assessment in cell lines, or (vi) the activity of tumor cells that create a proper milieu for cancer development.

Modulation of TLR9 levels probably occurs due to HPV16 E7 oncoprotein activity on the TLR9 promoter by interference in the NF-κB pathway. A chromatin repressive complex was also found on the TLR9 promoter, negatively regulating its transcription (Figure 3) [8]. A repression of the TLR9 expression disturbs the synthesis of IL-6, IL-8, and C-C chemokine ligand 20/macrophage inflammatory protein-3α (CCL20/MIP-3α) (which is an important chemokine for immune surveillance because of the recruitment of lymphocytes and Langerhans cells (LCs) to skin) [18]. The viral transcription and replication processes also undergo intervention, due to interferon deficiency caused by TLR9 repression [8]. Moreover, the altered levels of TLR9 can be caused by the devious expression of specific polymorphisms that change the effective receptor availability [19].

Other TLR expressions were also notably altered. In SCC (squamous cell carcinoma) specimens, it was demonstrated that the TLRs 3, 4, and 5 were significantly underexpressed while TLR1 was the only significantly overexpressed (TLRs 2, 7, 8, and 9 were not significant) when compared to the normal samples [13]. However, opposite results were also observed [3, 10, 11, 15, 16, 20]. No study has clearly demonstrated that altered TLR levels are a response of the host immune system against the infection or a consequence of virus activity supporting the infection. In addition, the knowledge of which cell is responsible for the alteration of TLR levels would be crucial to understand the TLRs' role in carcinogenesis; immune, stromal, and cancer cells have different functions in cancer development and thus, the shift of their TLR expression pattern could represent a marker for cancer progression or resolution [15].

Besides, another study found similar results and showed that the mRNA expression of TLR7 and 8 in cervical biopsies of cervical cancer patients was elevated [2]. In turn, infection regression (HPV16) was also associated with an increased expression of several TLRs (3, 7, 8, and 9), and their modulation could be used as a therapeutic approach [18].

Regarding other HPV-related cancers, TLRs were not as extensively studied like in cervical cancer. HPV is a key etiological factor of head and neck squamous cell carcinoma (HNSCC), in particular of oropharyngeal squamous cell carcinoma (OPSCC). HNSCC is commonly recognized as an immunosuppressive disease due to HPV activity. Consequently, an imbalanced cytokine profile, low amounts of $CD3^+$ $CD4^+$ and $CD8^+$ T lymphocytes, high infiltration of M2 macrophages (TAM), Treg cells and Treg/$CD8^+$ T cell ratio, impaired NK cell activity (higher expression levels of KIR genes), augmented expression of inhibitory receptors (e.g., CTLA-4, LAG-3, TIM-3, and PD-1), and decreased antigen presentation have been observed [21, 22].

Thereafter, the expression levels of several TLRs were reported and its dubious role was evident in some cases. It was found that TLR2 was upregulated in both vicinity

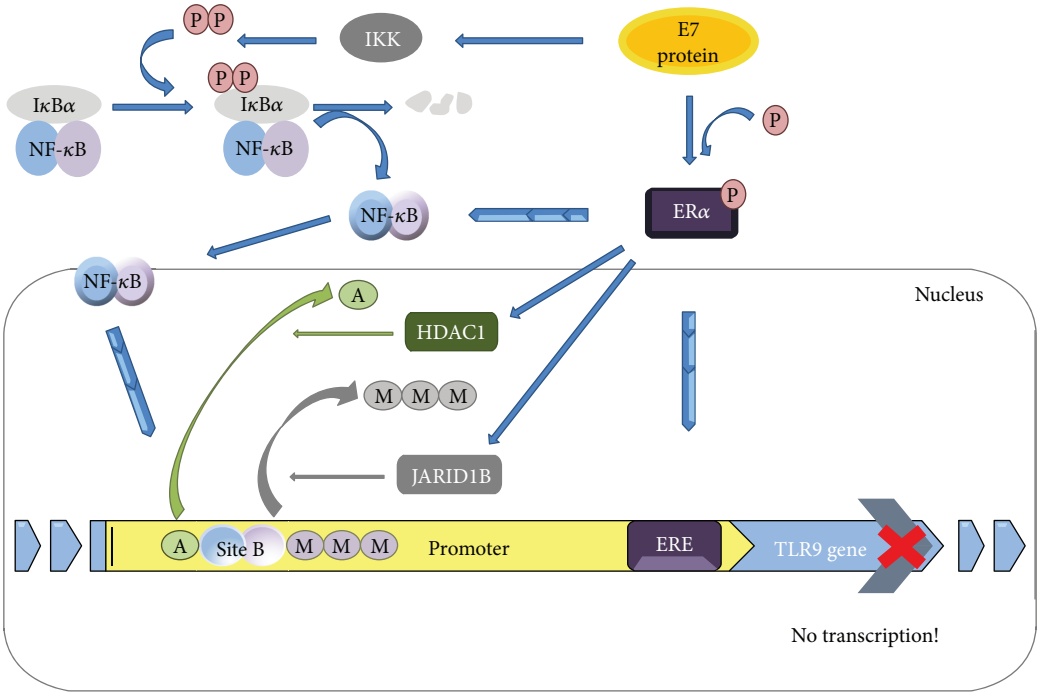

Figure 3: Inhibition of TLR9 expression by HPV16 E7 oncogene takes place via NF-κB canonical pathway, when this oncoprotein recruits the inhibitory complex NF-κB p50–p65 to a new *cis* element at the TLR9 promoter. This occurs with the additional binding of ERα (estrogen α) to another neighbour *cis* element, ERE (estrogen-responsive element), within that same promoter, and in the presence of HPV16 E7. ERα is also able to interact with the p65 subunit in the peri- or intranuclear region and contribute to transcription repression. Furthermore, it was also observed that there was a chromatin repressive complex composed by JARID1B demethylase and by HDAC1 deacetylase. These two catalytic units interact with ERα and negatively regulate TLR9 expression. The consequence of preventing TLR9 expression is the establishment of an immunosuppressive status with the inhibition of interferon and immune surveillance by cytokine responses [8]. NF-κB blue circle corresponds to p50 subunit and the purple one to p65. The straight blue arrows indicate an activation process or progress to the next stage; the curved arrows indicate motion; and the progressive arrows indicate the movement of some molecules interacting with the target. IKK: inhibitor of kappa B kinase; P: phosphate group; M: methyl group; A: adenyl group; JARID1B: lysine-specific demethylase 5B; HDAC1: histone deacetylase 1; Site B: 9-10 base pair DNA sites where p50 and p65 subunits bind.

immune cells and malignant keratinocytes in the microenvironment of oral squamous cell carcinoma (OSCC) compared to hyperplasia [23]. In another OSCC study, TLR3 was found to be upregulated in three head and neck cell lines (mRNA and protein), as well as when it induced apoptosis of the tumor cells, and *in vivo*, when it interrupted tumor growth. Also in this study, the mRNA expression of other TLRs was assessed observing elevated expressions of TLRs 1, 2, 4, and 6 while the TLRs 5, 7, 8, and 10 were found reduced [24]. In this type of carcinoma, however, TLR3 was found to have elevated levels and this event was attributed to an increase of tumor aggressiveness and invasion [25], a similar conclusion of another study which measured TLR3 in various HNSCC cell lines [26]. Both studies associated the triggering of the NF-κB pathway with the increase in tumor aggressiveness. In the mentioned studies above, it was not verified whether HPV was present or not.

TLR4 and 9 were also assessed in OSCC, and these receptors were found to have elevated expression levels which were correlated with tumor development [10]. Whether the increased levels of these receptors were associated with carcinogenesis or a reaction of the host immune response against tumor cells is still not known. TLR4 was also correlated with tumor development and protection of cancer cells from host immune response in an HNSCC study [27]. Several other studies have also reported controversial results regarding OSCC and HNSCC [10, 28], though it has been observed that different cell lines were used several times for the same purpose, which could explain the opposite results in some cases.

TLR7 was found overexpressed in the nuclear membrane and nuclei of cancer cells (a novel localization discovered) having higher levels in HPV-positive specimens, unlike TLR9 that showed reduced expression levels in HPV-positive samples compared to HPV-negative. TLR7 also showed altered localization depending on the cancer cell status: it was found with elevated levels in the plasma membrane of cancer-free cells compared to cancer cells, where this receptor was observed to have higher levels in the nuclear membrane and nuclei. Therefore, it seems that TLR7 may play different roles depending on the cancer cell status. Furthermore, increased levels in TLR7 demonstrated to be statistically significant for the direct correlation with p16. Thus, the increased levels of TLR7 could be indirectly related to E7 expression since the elevated levels of p16 are caused by the deregulation of pRb pathway, initially caused by E7 oncogenic activity, which probably also altered TLR9 levels. The other TLRs did not show any significant differences between cancer and control groups [29].

Based on what has been discussed above, the modulation of TLR expression or activities has been considered in the treatment of cervical and head and neck carcinomas as adjuvants in various vaccine strategies. The goal is to increase the synthesis of cytokines (e.g., IFN, TNF-α) and chemokines, generally by activating NK and dendritic cells, so as to activate CTL cells for generation of effector responses [30]. For a more efficient outcome, TLR agonists can be used in combination with non-TLR agonists, as demonstrated in a C57BL/6 mice tumor model in which HPV16 E7 vaccine was coadministrated with monophosphoryl lipid A (MPL), a TLR4 agonist, and α-galactosylceramide [7]. Also, TLR activities can be blocked aiming to hamper inflammation by preventing TLR downstream activation signalling, such as the MyD88-NF-κB pathway. Another interesting view to be highlighted is the importance of the modulation of TLR expression in stromal cells. It was found that these receptors were upregulated in these cells and may contribute to carcinogenesis [15].

Accordingly, several pharmacologic substances, which modify the activity of these receptors, have been tested/used first as adjuvants in vaccines for cervical cancer (e.g., Cervarix) and later in other HPV-related cancers. In the Cervarix vaccine for example, MPL is used to activate the innate response by interferon and proinflammatory cytokine synthesis. As a consequence, the adaptive response is also induced. Other examples are CpG (TLR9 agonist) alone or with rlipo (TLR2 agonist) [31], imiquimod and resiquimod (both TLR7 agonists), poly(I:C) (TLR3 agonist), and VTX-2337 (TLR8 agonist). Many studies have shown satisfactory results, especially with the simultaneous use of these agonists. Generally, an increased rate of tumor cell death upon TLR stimulation was observed [30]. Also, very high percentages of curing preexisting tumors in mice were reported [31–33].

In HPV-related cancers, the use of these adjuvants has also brought better outcomes. Imiquimod is able to induce Th1 responses by stimulating DC maturation and migration, Langerhans cell migration to draining lymph nodes, the inhibition of myeloid-derived suppressor cells, and the secretion of interleukins and cytokines [34]. In another study, imiquimod and poly(I:C) effects on cell death in *in vitro* and *in vivo* HNSCC models were evaluated. Both agonists were found to cause an increase in tumor cell death *in vitro* and *in vivo*. In addition, poly(I:C) induced higher levels of proinflammatory cytokine secretion (IFN-I, IL-6, and CXCL-10), MHC I expression of tumor cells, and monocyte activation *in vitro*, impairing tumor growth *in vitro* and *in vivo* even when TLR signalling was hampered on host cells [35]. Another study is currently recruiting participants to evaluate polyICLC (a modified version of poly(I:C)) combined with tremelimumab, a CTLA-4 antibody, and durvalumab, a PD-L1 antibody (clinicaltrials.gov identifier NCT02643303).

Another example is motolimod (VTX-2337), a TLR8 agonist which was tested for the treatment of HNSCC and other tumors. It was seen that this substance caused an increase in antitumor activities by inducing cytokine and chemokine synthesis as well as activating monocytes, NK, and dendritic cells, consequently boosting T cell activation [36, 37]. In a study using the TLR8 agonist with an anti-EGFR monoclonal antibody (cetuximab), an increase in NK cell-mediated cancer cell lysis and an enhancement of DC cross-priming of EGFR-specific $CD8^+$ T cells were observed [38]. Other possibilities are currently being tested for VTX-2337, such as combining it with cisplatin or carboplatin/fluorouracil/cetuximab (NCT01836029) and with cetuximab or cetuximab and nivolumab, an anti-PD-1 antibody (NCT02124850). In regard to TLR4, OK-432 (picibanil, approved in Japan) was tested in association with DCs and chemotherapy, and the results still have not been reported (NCT01149902) [30].

Other studies have also been or are currently being conducted with other TLRs: ISA201 (HESPECTA, a second generation vaccine based on ISA101), which uses a synthetic TLR2 agonist (Amplivant) with two HPV16 E6 peptides (NCT02821494) [38]; EMD 1201081 (IMO2055, TLR9 agonist) + cetuximab (NCT01040832), which did not demonstrate additional clinical efficacy than using cetuximab alone but it was well tolerated by patients [39]; EMD 1201081 + fluorouracil + cisplatin + cetuximab (NCT01360827); and entolimod (CBLB502), a TLR5 agonist which is being tested in combination with cisplatin and radiation (NCT01728480) due to the previously shown effects on radiation: it induced an increase in its therapeutic effect and a reduction of its toxicity *in vivo* when administered 1 h after exposure to radiation [40]. Table 1 summarizes the mentioned studies and is correlated with the Figure 4 which shows the therapeutic role and the activated signalling pathways of natural and synthetic TLR ligands.

3. Cytokines

Cytokines are the primordial mediators of the immune response, including antitumor activities. It has been reported that in cervical cancer as well as in HNSCC, immunostimulatory signals (Th1 cytokine profile) are hampered whereas proinflammatory and immunosuppressor ones (Th2 cytokine profile) are stimulated. Several studies have reported this shift of the cytokine pattern in preneoplastic and cancer specimens [41–43].

It is known that Th1 cytokines are potent activators of cellular-mediated immunity response that may precede HPV clearance, while Th2 cytokines impair the immune response, leading to an inefficient virus elimination and to chronic infection [43]. Furthermore, different hrHPV genotypes are associated with different cytokine profiles, so they interfere distinctly with the immune system, making disease progression different among the various hrHPV subtype infections [44]. Therefore, the modulation of cytokine expression is a key event for the induction of chronic infection and cancer development. It is known that the appropriate cytokine pattern defines the appropriate phenotype of immune cells, which whether or not results in the elimination of infected and (pre)cancerous cells.

Thus, cytokines were widely used in cancer immunotherapy, including cervical cancer and HNSCC. The main goal of their use was to induce a CTL response supporting the cancer cell apoptosis and tumor regression. They can be used in combination with several immunotherapy approaches such as DNA, DC-based and protein-based vaccines, TLR

Table 1: TLR-associated immunotherapy approaches for HNSCC treatment.

Number in Figure 4	Therapy	Therapy approach	Stage	Clinical trial identifier	Reference
1, 2	Imiquimod + poly(I:C)	TLR7 agonist + TLR3 agonist	*In vitro/ in vivo*	—	Klein et al. [35]
2	Poly(I:C)	TLR3 agonist + tremelimumab + durvalumab	Phase I/II	NCT02643303	No study was reported yet.
3	VTX-2337	TLR8 agonist	Phase I	NCT00688415	Dietsch et al. [37]
		TLR8 agonist + cetuximab	Phase I/II	NCT01334177	Stephenson et al. (2013) and Chow et al. [36]
		TLR8 agonist + cisplatin or +carboplatin/ fluorouracil/cetuximab	Phase II	NCT01836029	No study was reported yet.
4	OK-432 (Picibanil)	TLR4 agonist	Phase I	NCT01149902	Galluzzi et al. [30]
5	ISA201	TLR2 agonist + 2 HPV16 E6 peptides	Phase I	NCT02821494	Bann et al. [38]
6	EMD 1201081	TLR9 agonist which was tested with cetuximab	Phase II	NCT01040832	Ruzsa et al. [39]
		TLR9 agonist + fluorouracil + cisplatin + cetuximab	Phase I	NCT01360827	No study was reported yet.
7	CBLB502 (entolimod)	TLR5 agonist	Phase I	NCT01728480	Toshkov et al. [40]

agonists, and monoclonal antibodies (e.g., cetuximab and the immune checkpoint inhibitors like tremelimumab and durvalumab) [6, 34]. In the next subheadings, the most important cytokines for the treatment of HPV-related cancers are discussed according to their roles in the immune response.

3.1. Immunostimulatory Cytokines. Among the immunostimulatory cytokines, IL-2, IL-12, TNF-α, and interferons are the most prominent in anti-infection and antitumor activity. IL-12 is secreted by activated DCs and macrophages and is the most effective and promising cytokine for cervical cancer treatment. Several antitumor activities in animal models have been observed [34], such as the increase in IFN-γ and TNF-α levels, maturation of APCs and the lysis of immature ones, and the activation of NK responses (caused by the upregulation of NK activation receptors and ligands). Consequently, IL-12 stimulates Th1 polarization and CTL (antigen-specific response) cytotoxicity and plays an important antiangiogenic role [45].

As a result, IL-12 has been suggested to be used in several HPV-related cancer treatment strategies for potentiation of antitumor activity, for instance, in viral gene therapy, coadministrated with other cytokines or in DNA vaccine preparations [34, 38, 45], such as the INO-3112 vaccine. This promising strategy, which combines the gene sequences of E6 + E7 antigens (VGX-3100) and the IL-12 (INO-9012), has been tested for treatment of both cervical invasive (NCT02172911) and head and neck (NCT02163057) HPV-related cancers in clinical phase I/II trials with good results about safety and $CD8^+$ T cell immunogenicity [46].

Another ongoing study evaluated the immunotherapeutic effects of the coadministration of the recombinant IL-12 with cetuximab (NCT01468896) in patients with relapse, metastatic, or inoperable HNSCC. In the previous study regarding this combined treatment, an improvement in the lysis of tumor cell by NK cells was observed. In another approach using IL-12, a higher lymphocyte infiltrate and an improved overall survival rate were observed when IL-12 was coadministrated with IL-1β, IFN-γ, and TNF-α [47].

TNF-α is another key cytokine which creates an antitumoral milieu for virus elimination. This molecule supports the activation of macrophages, dendritic and NK cells and recruits them to the tumor site by inducing keratinocytes to release MIP-3α [18] and CCL2/MCP-1 (C-C motif chemokine ligand 2/monocyte chemoattractant protein-1), which is reduced in high-grade cervical lesions and in E6/E7-expressing cells [48]. Moreover, it inhibits HPV E6 and E7 oncogene transcription and proliferation in HPV-transformed keratinocytes *in vitro* [49] and causes apoptosis of cervical cancer cell lineage [50]. This cytokine is associated with lesion regression; its reduced level in serum and its presence in cervical cancer are correlated with tumor growth [51, 52]. Increased levels of TNF-α were also associated with CIN2/3 lesions, but also with an exacerbated inflammatory response [18]. A precise level of inflammatory response, which includes the precise secretion level of several cytokines including TNF-α, is the threshold between the occurrence and regression of cellular transformation. A sufficient level of this response at the infection onset is a mark of a valid immune response, but when the inflammatory response becomes excessive and persistent, it favours an appropriate milieu for infection development.

Along with TNF-α, interferon belongs to the group of crucial cytokines which creates an antiviral state, activating cell-mediated immunity which is potentiated in the presence of TNF-α (see Table 2 for IFN activities). Interferon is classified in type I (IFN-α and -β), type II (IFN-γ), and type III (IFN-λ) [62]. Similar to TNF-α, the amount of interferon

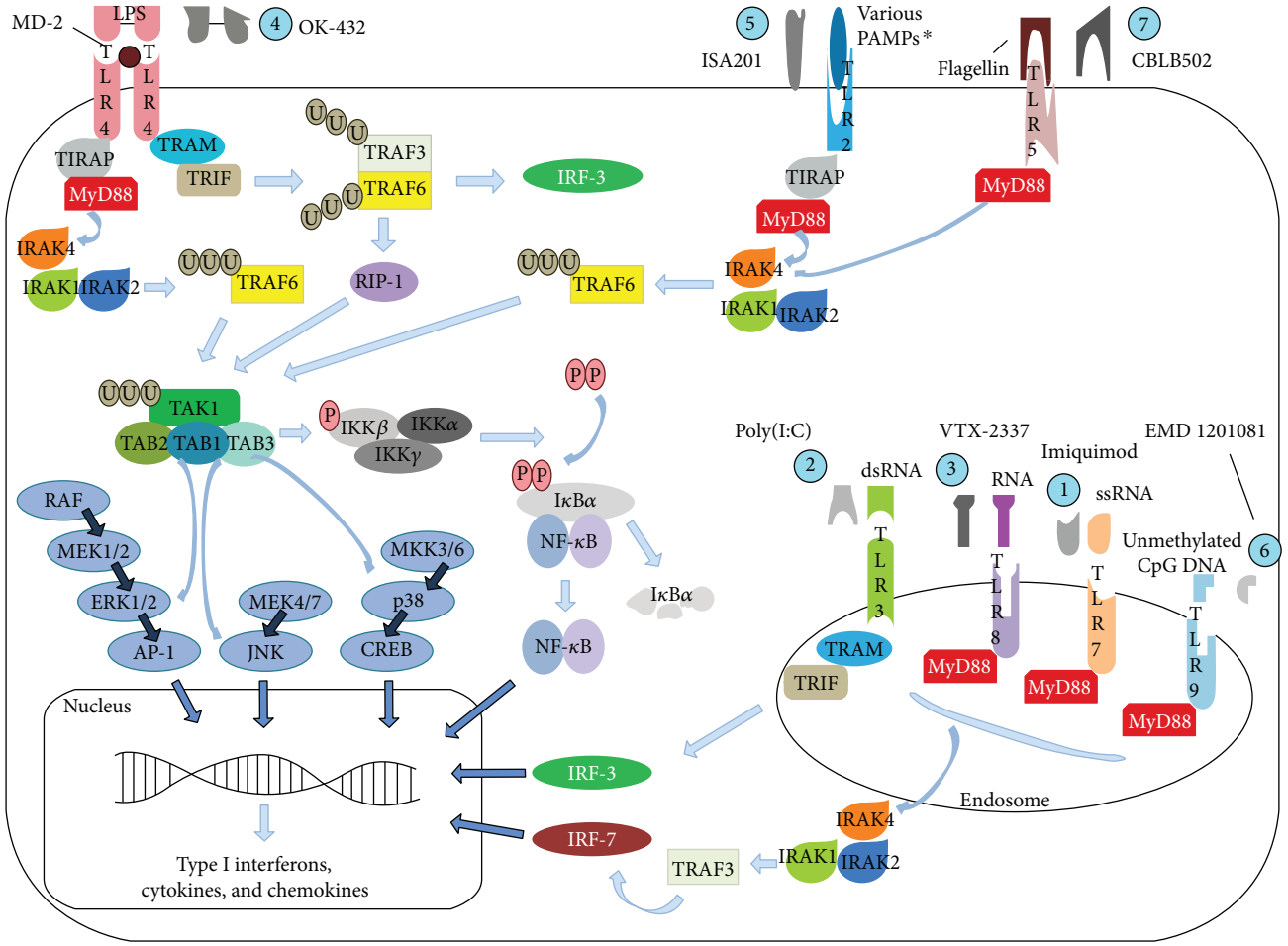

Figure 4: TLR signalling transducing pathways and the related immunotherapy approaches. Toll-like receptors are expressed in both immune and tumor cells, making difficult to decipher their function in cancer. Thus, their modulation in tumor microenvironment results from a complex balance of host and infected/tumor cell mechanisms which affects their expression and activation. They are activated by specific ligands (for each TLR) and synthetic substances as showed in the figure: (1) single-stranded RNA and imiquimod for TLR7, (2) double-stranded RNA and poly(I:C) for TLR3, (3) ssRNA and VTX-2337 for TLR8, (4) LPS, lipoteichoic acid, and Picibanil for TLR4, (5) *lipoproteins, peptidoglycans, lipoteichoic acids, zymosan, mannan, tGPI-mucin, and ISA201 for TLR2, (6) unmethylated dinucleotide cytosine-guanine and EMD 1201081 for TLR9, and (7) flagellin and CBLB502 for TLR5. Once activated, the signal transduction depends on adapter molecules such as MyD88, TIRAP, TRIF, and TRAM in order to activate the transcription of type I interferons and TLR-induced genes. All TLRs, except TLR3, require MyD88 for propagation of their signals. TLR4 requires myeloid differentiation factor-2 (MD-2) as a coactivator for its activation by LPS binding [3, 5]. TIRAP: TIR-domain-containing adaptor protein; MyD88: myeloid differentiation primary response protein 88; IRAK: IL-1R-associated kinase; TRAM: Toll receptor-associated molecule; TRIF: TIR-domain-containing adapter-inducing interferon-β; TRAF: TNF receptor-associated factor; RIP-1: receptor-interacting protein kinase-1; TAK: TNF receptor-associated factor; TAB: TGF-β-activated kinase I/MAP3K7-binding protein; IKK: inhibitor of kappa B kinase; IκBα: nuclear factor of kappa B, alpha; IRF: interferon regulatory factor; U: ubiquitination; P: phosphorylation.

arises mainly from NK cells [63], but T cells also synthesize these cytokines, both cytokines are upregulated in therapeutic approaches which cause infection regression [34].

With regard to this, it was suggested that the upregulation of IFN would be a good hallmark for HPV16 infection clearance [64]. This event is generally associated with the expansion of NK cell cytotoxicity [65] and the expression of IL-12 and TNF-α in infiltrated proinflammatory lymphocytes [57]. The increased levels of IFN restore immune function [55] and induce $CD4^+$ and $CD8^+$ responses, which led to a complete regression of the disease in a half of the patients who had undergone treatment. Several reports have shown the inhibition of type I IFN expression or their transduction pathways by HPV16 and 18 oncoproteins (E6 and E7). In this way, key genes involved in the immune surveillance and cytotoxic response are blocked [2, 56].

Diminished IFN-γ synthesis has also been associated with persistent infection and the level of malignancy of cervical lesions [66] as well as to the development of HPV-related cancer. In HNSCC, for example, the reduced levels of this cytokine are able to modify STAT1/STAT3 balance that blocks antigen presentation and DC maturation [47].

IFN-γ became a marker of the Th1-type antigen-specific cellular immune responses, involved in tumor resolution [67] and HPV clearance [18]. This cytokine induces: (i) synthesis of antiangiogenic factors (e.g., IP-10); (ii) cell cytotoxicity by

Table 2: IFN immune activities and HPV interferences.

IFN-I (IFN-α/β)	*Activities*
	IFN-α inhibits keratinocyte immortalization induced by HPV16 [53]
	Boosts IFN-γ secretion [54] and Th1 response [55]
	Induces antibody production [54]
	Induces resistance to viral replication [56]
	Induces MHC class I and II expression [54]
	Induces the activation of NK cells [56]
	Plays antiangiogenic and antiproliferative activities [57]
	Induces DC maturation and T cell proliferation and priming [54]
	Turns virus-infected cells more susceptible for CTL killing [56]
	Alters the B cell isotype and differentiation into plasma cell [54]
	Prevents T cell apoptosis [54]
	Promotes the proliferation of memory T cells [54]
	HPV interferences
	Its signal transduction pathways are prevented by E6 and E7 activities [18]
	IFN-α signalling is inhibited by (i) HPV18 E6 interaction with Tyk2 (tyrosine kinase 2), (ii) E6 binding to IRF-3 which prevents IFN-α transcription, and (iii) HPV16 E7 prevention of the displacement of p48 (subunit of interferon-stimulated gene factor 3 (ISGF3)) to the nucleus
	HPV16 E7 also inhibits IRF-1-mediated IFN-β transcription by physically interacting with IRF-1
	IFN-α and IFN-β are also downregulated by E6-mediated inhibition of STAT1 binding to ISRE and prevention STAT1 expression [57]
IFN-II (IFN-γ)	*Activities*
	IFN-γ as well as IFN-I inhibit transcription of E6/E7 genes in immortalized keratinocytes and malignant cells [48]
	Upregulates MHC class I in immune and tumor cells [58] as well as MHC I/II in epithelial cells [18]
	Promotes differentiation to the Th1 profile and induces these cells to produce IFN-γ [59]
	Plays antiviral, antiproliferative [58], and tumor cell killing activities, being associated with lesion regression [60]
	Boosts the synthesis of inducible nitric oxide synthase, IP-10 (protein 10 inducible by IFN-γ) and Mig (monocin inducible by IFN-γ) chemokines by macrophages, which augment cytotoxic response [58]
	Induces NK cell infiltration and activation [58]
	Induces TAP-1 (transporter antigen processing-1) and MCP-1 expression, which are important for T cell antigen recognition and chemoattraction, respectively [58]
	HPV interferences
	The expression of TAP-1 and MCP-1 is prevented by E7 and E6/E7, respectively [48]
IFN-III (IFN-λ)	*Activities*
	Prevents several tumor cell lines growth [61]
	Promotes antiviral and antitumor responses [61]
	In the treatment of viral and neoplastic diseases it has been tested with type I IFNs showing synergic effects and reduced side effects [61]
	HPV interferences
	None reported

boosting the overexpression of adhesion molecules; (iii) antigen presentation and synthesis of IL-12 by activation of DC; and (iv) more sensitivity to granule activity and death signalling [68]. In addition, type III IFN (IFN-λ) has also been demonstrated to play an important role in immune response against HPV infection. Alteration of this interferon was reported in hrHPV cell infection status, and it was suggested that it may support immune surveillance against HPV infection and cervical carcinogenesis. IFN-λ shows similar activities of type I IFNs but only specific cells respond to this type of IFNs like epithelial cells [62]. For all these activities and owing to the oncoprotein interference with these molecules, interferons are a class of cytokines with a great potential value when used in the development of more efficient approaches for HPV-related cancer therapy.

Another cytokine demonstrating to have strong antitumor activity is IL-2, which is produced primarily by $CD4^+$ cells after antigen priming. It plays several important roles, including the activation and maturation of DC, stimulation of NK cell cytotoxicity, the expansion of $CD8^+$ and $CD4^+$ cells, and the polarization for the Th1 cell profile [69]. Several studies have attributed the reduced levels of this cytokine to the lesion progression or cancer in HPV infection [21, 42, 70, 71]. Its reduced levels were suggested to be a viral evasion mechanism [71], which is frequently associated with increased levels of IL-10, TGF-β, and Treg cells [43]. Due to its strong immunoprotective activity, IL-2 has been used in several immunotherapeutic approaches, like in association with TG4001 HPV vaccine, which is constituted of a recombinant Ankara vaccinia virus expressing HPV16 E6 and E7 [6]. The disadvantages of IL-2 administration are systemic toxicity and the induction of Treg cell proliferation [69]. For example, in HNSCC, the administration of IL-2 and IFN-α2a demonstrated high toxicity [72].

3.2. Th17 Cells and Proinflammatory Cytokines. The inflammation process is an important feature of any cancer [1]. In regard to HPV-associated cancer, a deregulated proinflammatory network is induced by HPV inducing a favourable milieu for tumor development.

Th17 cell ($CD4^+$, IL-17^+), a T cell phenotype involved in the inflammatory response, was reported to be linked to the development of cervical cancer as well as others [73]. It has been shown that the percentage of this cell phenotype, as well as Th17/Treg ratio, was higher in peripheral blood samples of patients with premalignant and cervical cancer lesions when compared to the normal cytology group [74], a similar outcome previously observed [75]. In other studies, elevated levels of Th17 cells in CIN and cancer patients [73, 76] and in high-grade cervical lesions [77] were found, when compared to healthy controls. A higher statistical prevalence of this cell type was reported not only in serum but also in the cervical tissue of cancer patients. Likewise, a statistical significance of Th17 prevalence among patients with advanced (higher prevalence) and early stages in malignant processes was observed [78] and thus, its level has been deemed a good independent prognostic factor in cervical cancer [79].

In HNSCC, elevated levels of Th17 cells were found along with Treg [80] in serum and in tumor milieu, concluding its negative impact on the immune response against HPV, especially due to the induction of an exaggerated inflammatory response through IL-17 secretion [81]. The increased levels of Th17 or IL-17 were also found in patients with hypopharyngeal carcinoma, as well as other cancers such as colon, gastric and lung, suggesting a connection with cancinogenesis and malignant progression. Conversely, premalignant oral lesion treatments with TGF-β type I receptor inhibitor and IL-23 showed maintenance of the Th17 phenotype instead of changing to Treg cells. This resulted in the production of stimulatory and inflammatory molecules and slowed the progression of premalignant lesions to oral cancer [82]. The last research outcome demonstrated that the effect of Th17 is still unclear, mainly in other types of HPV-related cancers [79].

IL-1 (both IL-1α and -β) is the other interleukin present in higher levels in cervical cancer. Secretion of IL-1 is promoted by keratinocyte damage, the major IL-1-producing cells [83]. This interleukin is also secreted by TAM, another important cell in harmful proinflammatory response which induces metastasis, tumor growth, angiogenesis, and Treg differentiation [21]. IL-1 expression is modulated by NK-κB and vice versa, and the same occurs with TNF-α, that participates in the IL-1 synthesis pathway [83].

In particular, IL-1β exhibits an essential role in inflammation-associated carcinogenesis and supports tumor growth and metastasis. This interleukin promotes the secretion of a great range of cytokines, chemokines, growth factors, and various metastatic mediators, such as TGF-β, VEGF, metalloproteinases, and endothelial adhesion molecules [84]. In cancer studies, IL-1β is associated with a poor prognosis [84], and in cervical cancer research, it supports tumor progression and carcinogenesis [85]. IL-1β was also overexpressed in several types of tumors like breast, colon, oesophageal, lung, and oral cancer. A high throughput bioinformatics analysis plus *in vitro* and *in vivo* observation demonstrated that IL-1β is one of the key genes involved in HNSCC formation. This interleukin is closely related to the malignant transformation of oral cells, protumorigenic microenvironment generation that leads to oral carcinogenesis, and cell growth of the same type of cells [86]. Due to its crucial role in inflammation and carcinogenesis, this interleukin has been considered useful in therapeutic strategies [84] as well as IL-1α which also plays an important role in carcinogenesis [87]. Interestingly, the expression of IL-1α has been associated with higher risk of distant metastasis in HNSCC, the major cause of death in this type of cancer. In this scenario, the evaluation of IL-1α and clinical information may predict patients with high risk of HNSCC metastasis, thus leading to new treatment strategies [88].

Other examples of proinflammatory cytokines which support inflammation-associated cervical carcinogenesis are listed in Table 3.

3.3. Immunosuppressive Cytokines. There are several cytokines which are directly involved in downregulation of inflammatory status, promoting infection progress and cancer development. These include TGF-β, IL-4, IL-6, and IL-10 which are the main Th2 cytokines related to this anti-inflammatory profile and are discussed in this topic.

The expression of these cytokines is modulated by HPV oncoproteins o create a Th2 microenvironment. They have been reported to be upregulated in premalignant and cancer lesions [41, 52], and were suggested as biomarkers for HPV-related cancer [96]. Other Th2 interleukins are also encountered at high levels in cervical cancer patients, such as IL-9 [97] and IL-15 [44]. The latter and TGF-β, for example, were reported to induce the expression of CD94/NKG2A, preventing NK cell activity and CTL cytotoxicity [98]. An ongoing study evaluated a recombinant human IL-15 in advanced HNSCC patients in order to measure NK cell count, activity, and other immune response parameters (NCT01727076).

TGF-β plays a crucial role in the repression of immune responses against HPV and is upregulated in cervical

Table 3: Some proinflammatory cytokines in HPV-related carcinogenesis.

Cytokine	Action mechanism
IL-8	(i) It induces neutrophil chemoattraction and cell survival [18] (ii) It stimulates cell growth and cancer metastasis [89] (iii) Prognosis of patients with high levels of IL-8 is extremely poor [48] and its expression was associated with lesion severity [90]
IL-17	(i) It is associated with lymphatic metastasis [91] (ii) It is found in high levels in patients with cervical cancer [91] (iii) It is also linked to the antitumor response [92], when it supports the recruitment and activation of neutrophils, the maturation of DC/priming of T cells, and the synthesis of TNF-α, IL-1β, and IL-6 [74, 75, 93]
IL-23	(i) It is synthesized by activated APCs [94] (ii) It induces macrophage secretion of TNF-α, DC production of IL-12, and the synthesis of IL-17 [95] (iii) It induces upregulation of MMP9 (matrix metalloproteinase 9), tumor angiogenesis, and TAM activity and prevents T $CD8^+$ cell infiltration [95] (iv) As well as IL-17, shows antitumor activities through its immune surveillance properties, such as the promotion of CTL and NK cell, the IFN-γ secretion, and the stimulation of the IL-12-induced Th1 response [95]

carcinogenesis by viral oncoprotein activities. It blocks effector functions by suppressing antigen presentation, NK cytotoxicity, B cell and CTL proliferation, and cytokine synthesis of the Th1 profile. It downregulates IL-2 receptor signalling in T cells and IL-12 expression by APCs. Moreover, this cytokine is able to promote IL-10 expression by macrophages, induces proteolytic activity, which causes angiogenesis and metastasis [43], induces CD94/NKG2A expression on T cells [98], and stimulates the differentiation of T cells to Treg and Th17 phenotypes [74]. Moreover, TGF-β upregulation has been associated with favouring tumor development, CIN III specimens, cervical cancer, and cancer invasiveness [43].

IL-4 is another important cytokine frequently mentioned in cervical cancer studies. It is able to inhibit cytotoxic activity and IFN-γ expression, even in the presence of PMA (phorbol myristate acetate) and ionomycin—substances used in carcinogenesis models that stimulate immune responses [99]. This interleukin induces a switch to a Th2 responsiveness profile along with IL-2 [100] and is associated with viral persistence, disease severity, and progression of precancerous lesions [43, 100].

Like IL-4, IL-6 is upregulated during cervical carcinogenesis progression [90], playing a role in HPV-immortalized and carcinoma-derived cervical cell line proliferation [101]. It has been stated to induce the phosphorylation of STAT3 (the activated condition) in HNSCC, causing immunosuppression by inhibiting maturation of DC and activation of neutrophil, macrophage, NK and T cells. STAT3 is a key transcription factor which is involved in several other immunosuppressive activities such as IL-10 signalling, downregulation of IL-12, impairment of DC, and production of Treg cells [102]. IL-6 also contributes to the proliferation and inhibition of apoptosis of cancer cells, and thus, supports chronic inflammation and cancer development [51]. This interleukin affects DC migration, induces angiogenesis [103], MMP9 synthesis, and TAM differentiation [104]. However, IL-6 has also been reported to play anti-infection and antitumor functions [105]. Its expression is associated with a poor clinical prognostic factor [73] and its transcription repression can be used in immunotherapy approaches in cervical [106] and other HPV-related cancers [47].

IL-10 is the most studied Th2 cytokine with immunosuppressive activity in HPV-related cancer. It is synthesized by various cells, including Treg, Th2, M2 macrophages, APCs, and NK cells. This interleukin supports the creation of a microenvironment favourable to tumor development and it is the main cytokine having a Th2 role along with TGF-β. It hampers immune surveillance by blocking (i) antigen presentation by DC through the reduction of MHC II, adhesion, and costimulatory molecules, (ii) the synthesis of cytokines of the Th1 profile and (iii) the activities of monocytes and NK cells. IL-10 also supports immunomodulation by inducing the differentiation of T cells and macrophages to the Treg [43] and M2 profiles, respectively [107].

Furthermore, it has been demonstrated that IL-10 prejudices antiviral immune responses, since it impairs Th1 profile differentiation, $CD8^+$ cytotoxic response, and $CD3^+$ expression, which is essential in activating T cells. This interleukin also causes the downregulation of MHC I and II on the surface of monocytes. Moreover, this molecule is able to upregulate HPV16 E7 in cervical carcinoma cells *in vitro*, inducing tumor proliferation [43].

Despite the immunosuppressive activities cited above, there were disagreements over what high levels of IL-10 were related to, since it has been also associated with low grade lesions [66]. However, its immune regulatory activities have been well established. Elevated levels of IL-10 are commonly correlated with high-grade lesions and cancer condition, and its immunosuppressive roles have been reported countless

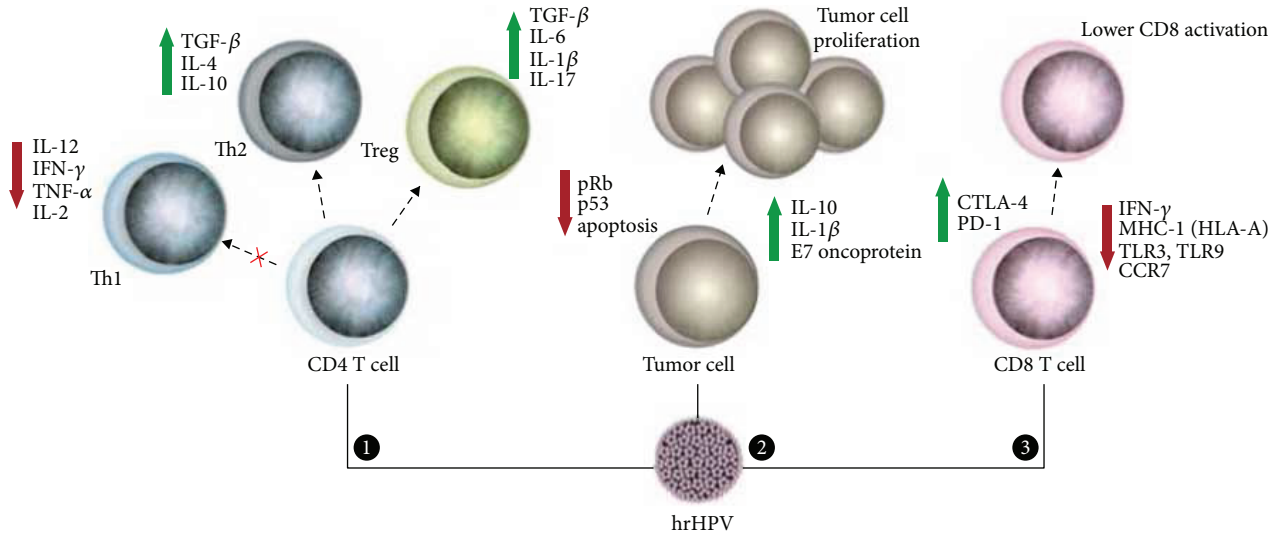

Figure 5: Interferences of HPV on $CD4^+/CD8^+$ lymphocyte responses, tumor proliferation, and apoptosis. (1) HPV is able to downregulate the Th1 response by decreasing proinflammatory cytokines. Moreover, Th2 and Treg response are stimulated by the virus because anti-inflammatory cytokines are expressed in higher amounts in tumor microenvironment. (2) Tumor cells show different mechanisms for their activation and survival. HPV (especially E6 and E7 oncogenes) promotes changes in cell cycle regulatory genes (e.g., pRb and p53), leading to tumor cell proliferation. In addition, Th2 cytokines produced by APC and T $CD4^+$ cells generate a chemical microenvironment that favours tumor establishment [2]. (3) T $CD8^+$ lymphocyte activities are affected by HPV infection that induces a decreased antigen presentation (MHC-I/HLA-A) and expression of TLRs and CCR7 on membrane surface of infected cells. Moreover, these cells also show upregulation of CTLA-4 and PD-1 inhibitor molecules [108].

times [42, 43, 52, 91], what are probably induced by HPV E2 protein activity [43].

Hence, IL-10, along with $CD8^+$ T cells and Treg cell ratio, has been considered an independent factor of poor prognosis. Treg cell is associated with tumor growth, tumorigenesis, and lymph node metastasis, constituting a poor prognosis for patients with cervical and other cancers as well [55]. These cells are rich in high-grade carcinoma samples, suppress CTL and NK cell activities, and support cancer progression through cytokine synthesis, such as IL-10 itself and TGF-β [43, 80]. Therefore, Treg cells, IL-10, and TGF-β (and other immune activities interfered by HPV are summarized in Figure 5) are considered targets for therapeutic interventions.

Another cytotoxic cell has also been encountered at high numbers in cervical cancer samples [109]. The expansion of this novel T cell subtype might affect lesion fate [110]. It is positive for CD4 and NKG2D markers and is subdivided in $CD28^{+/-}$, showing a statistically significant association with underexpression of several proinflammatory cytokines, such as IL1-β, IL-2, and TNF-α [109]. The role it plays and whether it is related to tumor growth or tumor suppression are still not known.

Novel cytokines in cervical cancer study have been gaining more and more attention due to their therapeutic potential. One of them, IL-37, has shown promising results because of its anticancer activity. For the first time, its anticancer role in an *in vitro* cervical cancer model has been demonstrated. This interleukin suppressed proliferation and invasion of HeLa and C33A cell lines, with higher inhibition rates in HeLa cell line, showing that anticancer activities were related to the HPV. This occurred through the inhibition of mRNA and protein expression of STAT3. Moreover, STAT3 phosphorylation was also blocked. This protein is a key transducer signalling molecule for developing an immune response in a tumor setting and could be an antagonist of IL-6 activity since STAT3 is central to the IL-6 signalling pathway [111].

In summary, cytokines are key molecules which modulate the pathology milieu. Thus, the regulation of the transcription, the synthesis, and the secretion of these signalling molecules in immunotherapies are necessary events to achieve satisfactory results in therapy. Cytokines are involved in several mechanisms of the immune system, such as immune cell maturation/differentiation, maintaining activation and regulating immune cell activities, which contribute to an immunoprotective background. Consequently, these molecules are important for therapy, i.e., by supporting the establishment of a proimmune milieu for infection clearance or by preventing the immunosuppressive role of immune cells. Many therapeutic interventions which take advantage of this have been developed and are currently in progress in therapeutic practices with very promising results, such as the active cytokine components of IRX-2, which caused an increase in antigen presentation, in NK cell activity, in the synthesis of costimulatory molecules and in $CD8^+$ T cell responses [112]. The mixture of cytokines is safe, generally well tolerated, and has currently been tested in a phase II trial for HNSCC [38].

4. Future Prospects

The immune response is vital in HPV-related cancer disease progression and resolution. In this set of collected data, the host immune response was observed to be used to benefit

patient's health through various ways, as these attributes occur in the natural resolution of infection. These new immunological approaches open novel horizons in diagnosis and especially in cancer therapy. TLRs and cytokines have been used to create an ideal tumor milieu for preventing tumor development or favouring transformed cell destruction. Their utilization appears to be a very promising immunotherapeutic strategy nowadays. The activation of DC and NK cells by means of administration of appropriate TLR and cytokines is essential to ensure the T-helper and cytotoxic responses.

Another possibility of using TLRs and cytokines in immunotherapy is their use in combination with monoclonal antibodies that prevent the activity of the immune checkpoint molecules, such as CTLA-4, PD-1, LAG-3, and TIM-3. These receptors are found in T cells and interact to ligands located at the cell membrane of tumor and antigen-presenting cells. In tumor pathogenesis, the activation of these molecules triggers signalling pathways which primarily prevent the function of $CD8^+$ and $CD4^+$ cells. In addition, the induction of other immune evasion activities offers a suitable environment for infection persistence and tumor development. Thus, inhibition of these activities may be considered a good therapeutic target; CTLA-4, for example, is found to be highly expressed in HPV-related cancer when compared to HPV-negative samples [22]. It competes with CD28 for interactions with the CD80 and CD86 ligands in DC surface, which prevented T cell priming by these cells. PD-1/PD-L1 is also greatly related to immune escape in HPV-related cancers, being correlated with a reduced disease survival rate due to CTL activity attenuation [102, 113]. Similarly, LAG-3 and TIM-3 have been recently considered in immunotherapy approaches. The first molecule enhances Treg cell activity [102]; and the second one induces T cell exhaustion and suppression of the innate response, associated with poor prognosis and tumor progression [114].

Checkpoint inhibitor monoclonal antibodies combined with other immune approaches have already been evaluated for cervical and HNSCC treatment: anti-PD-1 such as pembrolizumab (which is in ongoing studies for HNSCC treatment: NCT02255097 and NCT02252042) and nivolumab (NCT02105636 and NCT02488759); anti-PD-L1 such as durvalumab (NCT02207530) [46]; anti-CTLA4 such as ipilimumab and tremelimumab; anti-LAG-3 such as BMS-986016 (NCT01968109); and anti-TIM-3 (NCT02817633). The cited studies including LAG-3 and TIM-3 were for treatment of solid tumors. On the other hand, the combination with TLR agonists is new for HPV-related cancers; there are only two studies, NCT02643303 and NCT02124850, which are currently recruiting patients for testing the poly(I:C) with tremelimumab and durvalumab and the combination of VTX-2337 plus cetuximab or VTX-2337 plus cetuximab plus nivolumab, respectively. Regarding the evaluation of the combination of checkpoint inhibitors with cytokines in HPV-related cancers, no current study is reported at least to the best of our knowledge; and only three studies were reported for other HPV-unrelated solid tumors (NCT02614456, NCT02174172, and NCT02947165).

Therefore, the combination of different immunotherapeutic methods has shown increased beneficial effects and seems to be crucial in achieving better outcomes as observed in preclinical and clinical trials. As discussed here, HPV-related tumors require a great immune suppressor status for cancer development with increased activities of Treg, CTLA-4, and PD-1 and the suppression of APC and NK cells. Thus, studies on such evasion mechanisms are needed and offer new therapeutic perspectives.

Abbreviations

APC:	Antigen-presenting cell
CCL2/MCP-1:	C-C motif chemokine ligand 2/monocyte chemoattractant protein-1
CCL20/MIP-3α:	C-C motif chemokine ligand 20/macrophage inflammatory protein-3α
CCR7:	C-C motif chemokine receptor 7
CTL:	cytotoxic T lymphocyte (CD8 T lymphocyte)
CTLA-4:	Cytotoxic T lymphocyte-associated antigen 4
DAMP:	Damage- or danger-associated molecular patterns
DC:	Dendritic cell
EGFR:	Epidermal growth factor receptor
HDAC1:	Histone deacetylase 1
HeLa:	Human cervical cancer cell line
HNSCC:	Head and neck squamous cell carcinoma
HPV:	human papillomavirus
IκBα:	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha
IRAK:	IL-1R-associated kinase
IRF:	Interferon regulatory factor
ISGF3:	Interferon-stimulated gene factor 3
ISRE:	Interferon response element
IKK:	Inhibitor of kappa B kinase
JARID1B:	Lysine-specific demethylase 5B
LC:	Langerhans cell
LPS:	Lipopolysaccharides
MHC:	Major histocompatibility complex
MMP9:	Matrix metalloproteinase 9
MPL:	monophosphoryl lipid A
MyD88:	Myeloid differentiation primary response protein 88
NF-κB:	Nuclear transcription factor kappa B
NK:	Natural killer
OPSCC:	Oropharyngeal squamous cell carcinoma
OSCC:	oral squamous cell carcinoma
PAMPs:	Pathogen-associated molecular patterns
PD-1:	Programmed death-1 receptor
PD-L1:	Programmed death ligand-1
PMA:	phorbol myristate acetate
Poly(I:C):	Polyriboinosinic-polyribocytidylic acid
PolyICLC:	Modified version of poly(I:C) stabilized by lysine and carboxymethylcellulose
RIP-1:	Receptor interacting protein kinase-1

SCC: squamous cell carcinoma
TAB: TGF-β-activated kinase I/MAP3K7-binding protein
TAK: TNF receptor-associated factor
TAM: Tumor-associated macrophage (M2 macrophage phenotype)
TAP-1: Transporter antigen processing-1
TIRAP: TIR-associated protein
TNF-α: Tumor necrosis factor α
TLR: Toll-like receptor
TRAM: Toll receptor-associated molecule
TRAF: TNF receptor-associated factor
TRIF: TIR-domain-containing adapter-inducing interferon-β.

Conflicts of Interest

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

Authors' Contributions

Aldo Venuti and Antonio Carlos de Freitas equally contributed to this work.

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Research Article

Serum Sclerostin as a Possible Biomarker in Ankylosing Spondylitis: A Case-Control Study

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Objective. Several molecules are involved in the pathogenesis of a new bone formation in ankylosing spondylitis (AS). The aim of this study was to evaluate the serum levels of sclerostin in patients with AS as a possible biomarker and to investigate any correlations with radiographic damage, disease activity, and function. *Methods.* AS patients fulfilled the modified New York criteria, and healthy controls were enrolled for this study. BASDAI, ASDAS-CRP, BASMI, BASFI, patient and physician VAS, and C-reactive protein were evaluated at baseline visit. Spinal damage was assessed using the mSASSS on radiographs performed within 3 months from baseline. Serum concentrations of sclerostin were assessed at baseline and after four months of therapy in patients who started an anti-TNF. *Results.* Twenty healthy subjects and 40 AS patients were enrolled in the study. In our group, serum sclerostin levels (median (25th–75th percentile)) were significantly higher in healthy controls (18.04 (13.6–24) pg/ml) than in AS patients (6.46 (4.5–11.1) pg/ml; P value < 0.01). However, no significant correlations were found between serum sclerostin levels and radiographic damage, assessed by mSASSS, and between serum sclerostin levels and clinical indices of activity and disability or with laboratory parameters. Sclerostin levels did not show significant changes after 4 months of anti-TNF therapy. *Conclusions.* The results of our study suggest a possible role of sclerostin in the identification of AS patients. Further studies are needed to prove the role of sclerostin as a disease activity biomarker and progression of disease in AS.

1. Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease characterized by inflammation and new bone formation at axial and peripheral entheseal sites [1]. During the disease course, the development of syndesmophytes, enthesophytes, and spinal fusion is associated with chronic pain, functional impairment, and disability [1]. The introduction of biological therapies such as the inhibitors of tumor necrosis factor-alpha (TNF) and interleukin-17 has dramatically improved the overall outcome for patients with AS and non-radiographic axial spondyloarthritis [2–4] and shifted the focus of physicians towards the prevention of structural damage in the spine and other sites, to avoid loss of function and disability.

Several mechanisms involving cellular elements (i.e., osteocytes, chondrocytes, and immune cells), inflammatory cytokines, and cellular pathways seem to be responsible for new bone formation in AS. However, the precise mechanism in which inflammation and new bone formation are coupling is still not fully understood. Furthermore, the role of biologic treatments in the inhibition of radiographic progression is still almost unclear, even in the early stage of the disease [5–8]. Recently, the role of the Wnt/β-catenin pathway and its inhibitors, Dikkopf and sclerostin, has been evaluated in AS pathogenesis to identify a possible link with bone formation [9–11]. Wnt proteins bind to a receptor/coreceptor complex on the plasma membrane, which consists of LRP5/6 and Frizzled proteins. The engagement of this receptor complex by Wnt proteins leads to phosphorylation of β-catenin that

translocates in the nucleus where it is involved in the transcription of genes responsible for osteoblast differentiation and bone formation [12–14].

On these basis, it has been hypothesized that impaired expression of Wnt proteins or their inhibitors could contribute to the pathogenesis of AS. The study conducted by Appel et al. demonstrated the reduction of tissue expression of sclerostin at entheseal sites and in blood samples of AS patients compared with healthy subjects and patients affected by rheumatoid arthritis [9, 10]. Similarly, a reduction in serum levels of sclerostin and Dikkopf1 has been demonstrated in AS patients [5]. Thus, the molecules involved in Wnt/β-catenin could be possible useful biomarkers in AS.

The aim of this study was to evaluate the serum levels of sclerostin in patients with AS and to investigate any correlations with radiographic damage, disease activity, and function. Furthermore, a secondary aim was to evaluate the modifications of serum levels after treatment with anti-TNF agents.

2. Materials and Methods

The study was designed as a case-control study with a longitudinal part.

Patients affected by AS, referring to the Division of Rheumatology—Sapienza, University of Rome, and the Academic Rheumatology Unit, University of Molise, were consecutively enrolled in this study. Patients were classified according to the New York criteria [15]. Healthy subjects, matched by age and sex, were enrolled as a control group. Exclusion criteria were (1) age $\leq$ 18 years, (2) the presence of history of bone fractures in the previous 24 months, and (3) no treatment with bisphosphonate agents.

For all the patients enrolled in the present analysis, the following data were collected: demographic data, disease duration, extra-articular manifestations (EAM) (uveitis, inflammatory bowel diseases (IBD), and psoriasis), and clinical pattern (presence of peripheral arthritis, enthesitis, and dactylitis). All patients underwent a clinical assessment, and the following indices were evaluated:

(i) Swollen/tender joint count on 66 and 68 joints, respectively

(ii) Bath Ankylosing Spondylitis Metrology Index (BASMI) [16]

(iii) Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [17]

(iv) Bath Ankylosing Spondylitis Functional Index (BASFI) [18]

(v) Patient's visual analog scale (VAS) on global disease activity spinal pain (0–100 mm) [19]

The values of erythrocyte sedimentation rate (ESR, Westergren method, mm/h) and C-reactive protein (CRP, mg/l) were registered. The Ankylosing Spondylitis Disease Activity Score (ASDAS) was also calculated [20].

Radiographs of the spine and pelvis performed within three months from the enrollment in the study were collected in all patients. Each radiograph was evaluated by an expert reader (FMP) using the modified Stoke Ankylosing Spondylitis Spinal Score (mSASSS) [21]. According to the criteria of New York, the involvement of the sacroiliac joints was evaluated by assigning a score ranging from I (doubtful) to IV (complete ankyloses) [15].

2.1. Determination of Sclerostin Serum Levels. Serum samples of patients and controls were collected during the visits. Serum concentrations of sclerostin was assessed using commercial kit ELISA (*AUROGENE srl, Rome, Italy*). The serum samples of patients were taken at the time of the visit and stored at −80°.

AS patients, naïve to biologic treatment who started biologic therapy (anti-TNF) due to high disease activity, were longitudinally followed up for four months. In this subgroup of patients, sclerostin serum concentrations were evaluated at baseline (sample taken the day of starting anti-TNF therapy) and after four months of anti-TNF therapy.

2.2. Statistical Analysis. Statistical analysis was performed using the PRISM program 5—GraphPad. Normally distributed variables were summarized using the mean ± standard deviation (SD) and nonnormally distributed variables by the median/25th–75th percentile. Percentages were used when appropriate. Mann–Whitney test was performed for unpaired categorical data and *t*-test for paired samples. Univariate comparisons between nominal variables were calculated using chi-square test or Fisher's test where appropriate. The significance of the correlation was assessed by the correlation coefficient of Spearman's rank. Two-tailed *P* values were reported. *P* values less than 0.05 were considered significant.

3. Results

During one-year period, we enrolled 40 AS patients and 20 age- and sex-matched controls. Of the 40 AS patients, 15 subjects who started anti-TNF treatment were prospectively followed up for 4 months. The main demographic, clinical, laboratory, and X-ray findings of AS patients enrolled in the present study were summarized in Table 1. Peripheral involvement was registered in 14 patients (35%). Most of the patients had inactive or moderate disease activity (median ASDAS-CRP: 2.1). The serum concentrations of sclerostin in AS patients and in healthy controls were described in Figure 1. In our group, serum sclerostin levels (median (25th–75th percentile) were significantly higher in healthy controls (18.04 (13.6–24) pg/ml) than in AS patients (6.46 (4.5–11.1) pg/ml; *P* value <0.01) (Figure 1). Figure 2 shows the receiver operating characteristic (ROC) curve for serum levels of sclerostin in patients with AS. Serum levels $\leq$ 13 pg/ml have sensitivity and specificity of 82.5 and 90%, respectively, with a likelihood ratio of 8.25. Furthermore, sclerostin serum levels, although without a significant difference, were found to be lower (4.2 (3.1–5.9) pg/ml) in patients with ankylosis of sacroiliac joints (grade IV) than

Table 1: The main demographic clinical and X-ray features of AS patients ($n = 40$).

Male/female	30/10
Age (median/25th–75th percentile) year	50 (40.5–56.75)
Disease duration (median/25th–75th percentile) year	12.5 (6.2–21.5)
HLA-B27, *n* (%)	28 (70)
CRP, mg/dl (median/25th–75th percentile)	0.5 (0.2–0.9)
ESR mm/hr (median/25th–75th percentile)	12.5 (5–20.7)
VAS global health (median/25th–75th percentile)	4.75 (3–5.9)
VAS physician (median/25th–75th percentile)	4 3–5
ASDAS-CRP (median/25th–75th percentile)	2.1 (1.5–3.3)
ASDAS-ESR (median/25th–75th percentile)	2.2 (1.4–3.25)
BASDAI (median/25th–75th percentile)	3.65 (2–5.2)
BASMI (median/25th–75th percentile)	2 (1–5)
BASFI (median/25th–75th percentile)	1.65 (1–3.9)
Sacroileitis IV grade, *n* (%)	14 (35)
Sacroileitis II-III grade, *n* (%)	26 (65)
mSASSS (median/25th–75th percentile)	10 (2.5–29.5)
Peripheral involvement (%)	14 (35)
Enthesitis, *n* (%)	12 (30)
Psoriasis, *n* (%)	3 (7.5)
Inflammatory bowel disease, *n* (%)	5 (12.5)
Uveitis, *n* (%)	9 (22.5)
Treatment, *n* (%)	
NSAIDs	17 (42.5)
DMARDs	6 (15)
Anti-TNF	22 (55)

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; VAS: visual analogic scale; ASDAS: Ankylosing Spondylitis Disease Activity Score; BASMI: Bath Ankylosing Spondylitis Metrology Index; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; mSASSS: Stoke Ankylosing Spondylitis Spinal Score: DMARDs: disease-modifying antirheumatic drugs; NSAIDs: nonsteroidal anti-inflammatory drugs; TNF: tumor necrosis factor.

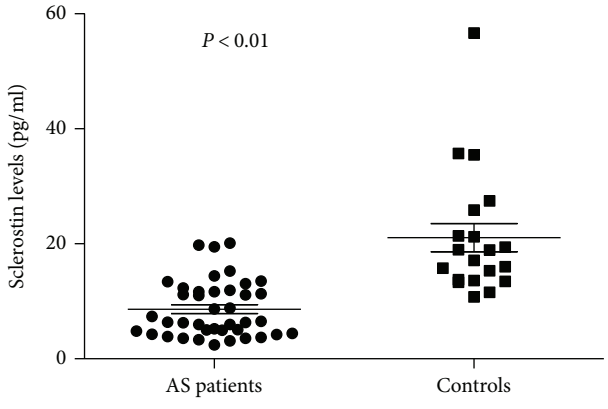

Figure 1: Sclerostin serum levels in patients with AS ($n = 40$) and in healthy controls ($n = 20$).

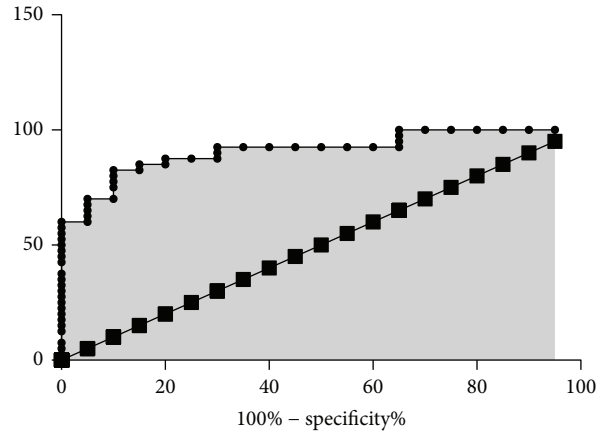

Figure 2: Receiver operating characteristic (ROC) curve for serum levels of sclerostin in patients with AS ($N = 40$) and in healthy controls ($n = 20$). Area under the curve: 0.91; 95% confidence interval: 0.8377 to 0.9823; P value: <0.01.

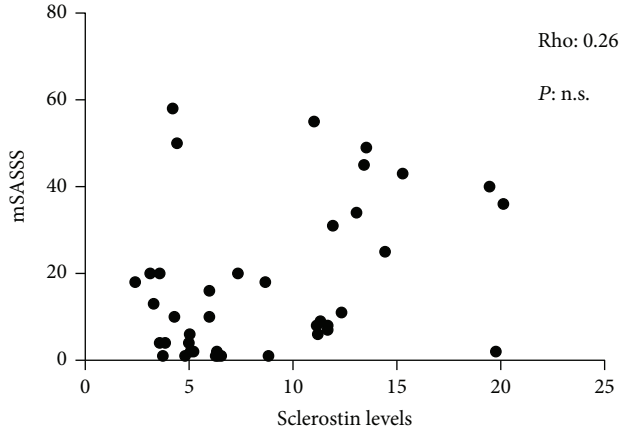

Figure 3: Correlation (Spearman's rho) between serum levels of sclerostin and mSASSS in patients with AS. mSASSS: modified Stoke Ankylosing Spondylitis Spinal Score.

in patients with grade II and III sacroiliitis (5.07 (4.07–6.4) pg/ml, $P = 0.07$). However, no significant correlation was found between serum sclerostin levels and radiographic damage, assessed by mSASSS (Figure 3), and no significant correlations were found between serum sclerostin levels and clinical indices of activity and disability or with laboratory parameters.

Sclerostin levels did not show significant changes after 4 months of anti-TNF therapy in the 15 prospectively evaluated patients (Figure 4).

4. Discussion

In the present study, we evaluated the possible role as a biomarker of serum level of sclerostin in a cohort of patients affected by AS.

The understanding of AS pathogenesis is a critical issue in order to prevent the bone formation, probably the most important cause of disability and reduced quality of life in AS patients. In the last years, several new players involved

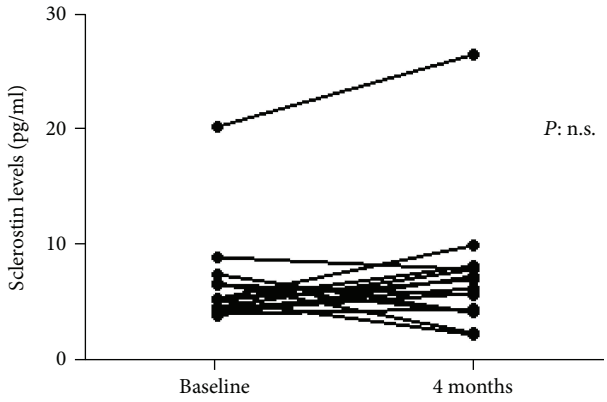

Figure 4: Sclerostin serum levels in patients with AS ($n = 15$) who started an anti-TNF due to high disease activity before (baseline) and after 4 months of treatment.

in the AS pathogenic mechanisms have been suggested, with encouraging results. In our study, we confirmed the presence of significantly lower serum levels of sclerostin in patients with AS compared to healthy controls. This result is in keeping with the previous studies published in the literature. In particular, Appel et al. showed significantly lower serum sclerostin levels in a cohort of 46 patients with AS compared to healthy subjects and patients with osteoarthritis. Serum sclerostin levels were also significantly associated with the development of new 1-year and 2-year follow-up syndesmophytes, reinforcing the hypothesis that the lower expression of Wnt inhibitors contributes to the activation and differentiation of osteoblastic cells [9]. In the same study, the authors demonstrated a reduced local expression of sclerostin at the level of bone tissue samples obtained from patients with AS [9]. These results were confirmed in other studies evaluating AS patients [22, 23].

In the study conducted by Saad and colleagues, sclerostin levels increased after 6 months and 12 months of anti-TNF treatment. Furthermore, the authors themselves reported persistently reduced sclerostin levels in patients with a high disease activity [22].

In our study, sclerostin levels ≤ 13 pg/ml showed sensitivity and specificity of 82.5 and 90%, respectively. However, this high discriminative capacity works only if compared with healthy controls and further analysis should be performed, in particular, in patients affected by disease belonging to the spondyloarthritis group such as psoriatic arthritis, or in patients with osteoarthritis. In our group, we did not find any correlations with disease activity; this result could suggest that inflammation and sclerostin levels might be unrelated. Furthermore, considering the group of 15 prospectively patients evaluated, no significant differences were found between serum sclerostin levels at baseline and after 4 months of anti-TNF treatment, although there was a tendency towards increasing levels after treatment. According to the study conducted by Saad and collaborators, this could be linked to the short duration of follow-up in our patients, to the small number of patients or to the hypothesis that other cytokines or cellular pathways may play a role in the suppression of sclerostin expression. Recently, a systematic review did not suggest the role of sclerostin as a potential biomarker, due to the lack of significant differences in serum levels of sclerostin between cases and controls [24]. However, several limitations should be considered in this meta-analysis. First, there are very few studies and a relative small number of patients; thus, the limited size might affect the conclusion. Second, the articles, which only support median and range, were excluded [24].

The results of our study are difficult to interpret. We did not find any significant association between sclerostin levels, disease activity, and radiographic damage. This result reinforces the hypothesis that inflammation and new bone formation are paired, but independent processes and sclerostin levels are not consequently influenced by the presence of inflammation, the duration of disease, or disease activity. It can be hypothesized that the pathogenetic processes that cause a decrease in sclerostin levels could occur early and autonomously self-sustain. However, the very low serum sclerostin levels found in AS patients in respect to controls reinforce the hypothesis of its involvement in AS pathogenesis.

5. Conclusions

The results of our study suggest a possible role of sclerostin in the identification of AS patients. However, further studies are needed to prove the role of sclerostin as a disease activity biomarker and progression of disease in AS.

Data Availability

Databases are stored at University of Molise, Department of Medicine and Health Sciences.

Conflicts of Interest

Authors declare no conflict of interest.

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Research Article

A Cross-Sectional Study of the Association between Autoantibodies and Qualitative Ultrasound Index of Bone in an Elderly Sample without Clinical Autoimmune Disease

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Bone loss is characteristic of the ageing process and a common complication of many autoimmune diseases. Research has highlighted a potential role of autoantibodies in pathologic bone loss. The confounding effects of immunomodulatory drugs make it difficult to establish the contribution of autoantibodies amongst autoimmune disease sufferers. We attempted to examine the relationship between autoantibodies and bone mass in a population of 2812 elderly participants without clinical autoimmune disease. Serum samples were assayed for a panel of autoantibodies (anti-nuclear, extractable nuclear antigen, anti-neutrophil cytoplasmic, thyroid peroxidase, tissue transglutaminase, anti-cardiolipin, rheumatoid factor, and cyclic citrullinated peptide). Bone mass was measured using quantitative ultrasound (QUS) of the calcaneus. The relationship between each autoantibody and bone mass was determined using linear regression models. Anti-nuclear autoantibodies were the most prevalent, positive in approximately 11%, and borderline in roughly 23% of our sample. They were also the only autoantibody observed to be significantly associated with QUS index in the univariate analysis ($n = 1628$; $r = -0.20$; 95% CI: −0.40–0.00; $p = 0.046$). However, statistical significance was lost after adjustment for various other potential confounders. None of the other autoantibodies was associated with QUS index in either univariate or multivariate analysis. We are limited by the cross-sectional nature of the study and the low prevalence of autoantibodies in our nonclinical sample.

1. Introduction

Normal bone remodelling requires a tight coupling of bone resorption to bone formation, whereby the two processes occur simultaneously and in harmony to guarantee negligible change in bone mass and therefore no alteration in bone quantity after each remodelling [1]. Loss of coupling between these two processes leads to osteoporosis, a combination of pathologic bone loss and altered microarchitecture that results in fragility fractures in response to minimal or low velocity force [2, 3]. Osteoporosis is predominantly a condition of the elderly and accounts for approximately 2 million fractures annually, including hip, vertebral (spinal), wrist, and other fractures [3, 4]. Osteoporotic fractures contribute

to a marked increase in morbidity and mortality, as well as health care costs and disability amongst this cohort [3].

In recent years, pathologic bone loss has been associated with disorders characterised by immune dysfunction, hinting at the presence of an immune-skeletal interface [5, 6]. Research aimed at elaborating this relationship has identified common cell types and shared mediators that play functional roles in both systems [7]. For example, vitamin D, parathyroid hormone (PTH), testosterone, and leptin, all recognized regulators of bone function, are also acknowledged to modulate immune function [8]. Additionally, macrophages, osteoclasts, and dendritic cells are all derived from the same myeloid precursors, with the latter two noted to exhibit the same lifecycle [9]. Notably, varieties of immune cells have been observed to regulate osteoclast and osteoblast activity in turn mediating the process of bone remodelling, responsible for maintaining the quality of the skeleton [10]. It therefore stands to reason that perturbations in the immune system would translate into disruptions in bone homeostasis.

The emergence of the field of osteoimmunology has recognized the immune system as a vital player in fine-tuning the balance between bone resorption and bone formation [11]. Though the role of inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin- (IL-) 1β, and IL-6 in enhancing osteoclast-mediated bone resorption is well established, the involvement of autoantibodies is still poorly defined [12]. Nevertheless, the findings of our recent literature review provided strong evidence justifying further research aimed at delineating the relationship between autoantibodies and bone mineral density (BMD) [13]. Autoantibodies against a myriad of antigens have been linked to pathologic bone loss [14–20]. For instance, autoantibodies against rheumatoid factor (RHF) and anti-cyclic citrullinated peptide autoantibodies (anti-CCP) have been identified as independent risk factors for the development of bone erosion and osteoporosis in rheumatoid arthritis (RA) [14–17]. Similarly, autoantibodies targeting tissue transglutaminase (anti-TTG) have been significantly associated with a higher risk of hip fracture and reduced BMD levels in celiac disease [18, 19]. Moreover, anti-nuclear autoantibodies (ANAs) have been implicated in reduced BMD in systemic lupus erythematosus (SLE) populations [20]. These findings were however not confirmed by all studies [21, 22].

Notably, many of the studies attempting to characterise the relationship between autoantibodies and bone mass have been conducted in clinical populations with autoimmune conditions [15–28], where there are confounding effects of immunomodulatory drugs such as steroids, which are themselves implicated in pathologic bone loss. Studies examining the usefulness of autoantibodies as a method of screening the general population for osteoporosis are few and contradictory. Our literature review identified a single study utilising middle-aged women drawn from the general population that failed to verify previous findings that autoantibodies against osteoprotegerin (OPG) correlate with BMD [29]. Alternatively, studies comprising samples without any gastrointestinal symptoms of celiac disease, villous atrophy, or evidence of malabsorption reported significant associations between low BMD and increased circulating concentrations of autoantibodies against TTG and EMA (anti endomysial autoantibodies) [23–25]. Furthermore, the clinical utility of autoantibodies in relation to monitoring BMD variability remains an important research question on account of the increasing body of research. Studies utilising clinically autoimmune samples reporting observations of a significant association between a range of autoantibodies and BMD remain important observations given these associations remained significant even after adjusting for age, gender, body mass index (BMI), vitamin D, and smoking habits, all important mediators of bone mass [17]. Moreover, studies utilising early arthritis patients prior to the clinical onset of disease and before the start of treatment have further demonstrated a potential role for autoantibodies in mediating bone health through observations of significant associations between autoantibodies such as anti-CCP and anti-RF and decreased BMD independent of disease activity, specifically inflammatory status [26–28]. Furthermore, animal models have documented the ability of autoantibodies to directly upregulate bone resorption, providing compelling support for a causative role of these antibodies in pathologic bone loss [30–32].

Quantitative ultrasound (QUS) is a noninvasive ultrasound-based technique useful in assessing bone structure (elasticity and microarchitecture) as well as bone mass, therefore providing determinations of bone quality beyond those associated with the dual-energy X-ray absorptiometry (DEXA) [33]. The latter technique is currently considered the gold standard and comprises an accurate technique used to measure BMD at specific fracture-related sites, namely, the spine, hip, and radius [33]. Recently, research has acknowledged the role of bone microarchitecture and elasticity in conjunction with bone density in the development of bone fragility and subsequent fractures, in turn highlighting the important contribution of the QUS measurements [34]. The widespread interest in QUS as a useful measurement tool is also increasingly attributed to it being a rapid, portable, and radiation-free technique making it a more suitable alternative to DEXA for screening a large sample of relatively healthy elderly subjects [35–42]. Additionally, QUS measurements of bone property have been illustrated to correlate with real and volumetric BMD measured by DEXA [37, 38, 40, 42–48]. In this study, we shall take advantage of heel QUS to assess bone properties of our healthy elderly sample.

Age is an important risk factor for autoimmunity, with several autoimmune diseases preferentially occurring more prevalently in the second half of adulthood [49, 50]. Moreover, several studies have reported a higher prevalence of both organ and non-organ-specific autoantibodies amongst the elderly even in the absence of autoimmune disease when compared to the general population [49]. Of particular interest is the fact that the elderly represent a population characterised by high levels of autoantibodies that do not necessarily reflect clinical autoimmune disease [50]. We were therefore able to take advantage of the latter characteristic to evaluate the association between autoantibodies and bone mass in a population without clinical autoimmune disease in order to eliminate the confounding effect of immunomodulatory drugs.

Osteoporosis is one of the most prominent worldwide public health problems, and morbidity is increasing with the ageing global population [51]. As a silent disease without obvious symptoms and evidence until fracture, early diagnosis remains the key strategy to enable efficient management of this condition. Research has successfully demonstrated the benefits associated with early diagnosis and subsequent intervention on the delayed progression of the disease as well as improved outcomes [52]. Therefore, searching out biomarkers that are able to identify individuals at high risk of developing osteoporosis particularly at a time when BMD measurements of DEXA or QUS do not offer enough information to make a diagnosis would prove particularly useful.

2. Materials and Methods

2.1. Ethical Statement. This research was approved by the Human Research Ethics Committees of the Hunter New England Health District and the University of Newcastle (03/12/10/3.26).

2.2. Population. Participants were drawn from the Hunter Community Study (HCS), a longitudinal study comprising a cohort of 3318 community-dwelling Australians aged 55–85 years, randomly selected from the New South Wales (NSW) electoral roll. The specifics of their recruitment and characteristics have previously been described [53].

Individuals with clinical autoimmune disorders were excluded from this analysis (Table 1). These exclusions were instituted to investigate the link between autoantibody and bone mass in a nonclinical sample in order to eliminate the confounding effect of immunomodulatory medications associated with pathologic bone loss. Study participants with osteoporosis were additionally excluded from this analysis.

2.3. Data Collection. At baseline, study participants completed a range of postal questionnaires covering a wide range of data such as medical history, smoking habits, medication use, and dietary habits. Self-report questionnaires were returned by participants when they attended the HCS data collection clinic during which time blood samples were collected and included plasma, serum, whole blood, and DNA that was stored at 80 degrees Celsius in 1 mL aliquots to minimize freeze-thaw cycles. Additional clinical measures obtained at the data collection center included BMI, level of physical activity, and QUS measurements. Study participants also consented to the linkage of their HCS study data to local and national information databases and records (Table 2). Linking of HCS data to these databases provided a range of detailed information including data on the use of prescription medication.

2.4. Autoantibody Measurements. Serum autoantibody titres were determined using a variety of assays.

(i) HEp-2 ANA slides supplied by Kallestad (Bio-Rad Laboratories) were used to measure anti-nuclear autoantibody (ANA) titre. Negative, borderline, and positive categories corresponded to titres $< 1:40$, $1:40 \geq$ titres $\leq 1:80$, and titres $\geq 1:160$, respectively.

Table 1: Autoimmune conditions and chronic diseases for exclusion.

ANA related
Lupus, SLE
Sjogren's syndrome
Scleroderma, CREST
Myositis: polymyositis, dermatomyositis
Autoimmune liver disease
Primary biliary cirrhosis
Polyarteritis nodosa
Cancer now (malignancy in last 5 years approximately)
Cancer ever (only melanoma included from skin cancers)
(Active) chronic infection (hepatitis A, hepatitis C)
ANCA related
Vasculitis
Wegener's
Churg-Strauss
Polyangiitis: microscopic polyangiitis, granulomatosis polyangiitis, eosinophilic granulomatosis with polyangiitis colitis
IBD: inflammatory bowel disease (not irritable bowel syndrome: IBS)
ACGA related
Antiphosholipid syndrome
Lupus inhibitor
RHF/CCP-Ab related
Rheumatoid arthritis
Psoriatic arthritis
Ankylosing spondylitis
TTG-Ab related
Celiac disease
TPO-Ab related
Thyroid disease: Grave's disease, Hashimoto's disease
Chronic infections
Glandular fever
Ross river virus
Malaria
Dengue

Table 2: Local and national health information databases linked to hunter community study data.

(i) Hunter New England Area Allied Health Records
(ii) Hunter New England Area Health Service Community Service Records
(iii) Hunter New England Area Health Service Outpatient Records
(iv) Hunter New England Area Health Service Hospital Admission Records
(v) Hunter New England Area Heart and Stroke Register and Diabetes Register
(vi) New South Wales Cancer Registry
(vii) Medicare Australia and Pharmaceutical Benefits Scheme

(a) Individuals classified as borderline or positive for ANA were subsequently tested for extractable nuclear antigen antibodies (ENA) using an enzyme-linked immunosorbent assay (ELISA). Samples were screened for 6 antigens, namely, anti-Smith (Sm), ribonucleoprotein (RNP), Sjogren's syndrome A and B (SSA and SSB), topoisomerase I (SCL-70), and autoantibodies against amino acyl-tRNA synthetases (Jo-1) (Immuno-Concepts, USA). Individuals who tested positive for ENA but had no defined antigen specificity identified were classified as borderline for ENA, whilst those who had at least one of the six antibody specificities identified were classified as positive.

(ii) Commercial formalin-fixed neutrophil slides (INOVA Diagnostics Inc., San Diego, California) were used to measure anti-neutrophil cytoplasmic antibodies (ANCA). Indeterminate and atypical ANCA was categorised as borderline whilst those staining with a cytoplasmic fluorescence of classical cytoplasmic or perinuclear pattern of 1 : 10 or higher dilution were classified as positive.

(iii) ELISA (Aesku, Germany) was used to test for thyroid peroxidase (TPO) autoantibody titres. Titres ≥ 50 units per millilitre (units/mL) were deemed positive.

(iv) AESKULISA CeliCheck immunoglobulin A (IgA) and immunoglobulin G (IgG) tissue transglutaminase (TTG) ELISA (six-point calibrator) was used to measure TTG autoantibody titres where titres ≥ 25 units/mL were considered positive.

(v) ELISA produced by Medical Innovations (four-point calibrator curve) was used to test for anti-cardiolipin antibodies (ACGA). Titres ≤ 5 IgG phospholipid units (GPL) were defined as negative whilst 6–20 GPL was low positive, 21–40 GPL moderate positive, and over 40 GPL high positive.

(vi) Rheumatoid factor (RHF) was measured using the RHF Beckman Coulter Immage Immunochemistry system, and titres ≥ 20 international unit per millilitre (IU/mL) were defined as positive.

(vii) Anti-cyclic citrullinated peptide antibodies (anti-CCP) were measured using an anti-CCP2 kit QUANTA lite (INOVA Diagnostics Inc., San Diego) with titres > 20 enzyme immunoassay units per millilitre (EU/mL) defined as positive.

All ELISA were performed on the Grifols Triturus platform (Grifols USA, LLC).

2.5. Outcome Measure. Bone mass was measured at the heel using quantitative ultrasound (QUS) (Sahara Hologic sonometer, Hologic Inc., MA, USA). The calcaneus (heel bone) is a recognized and preferred peripheral site for assessing bone quality because it has high metabolic turnover rate and possesses two lateral surfaces, which facilitate the movement of ultrasound through the bone [54]. The heel bone also contains a large percentage of trabecular bone (~95%), which has a high metabolic turnover and a pattern of bone loss similar to the spine [55–57]. Results were expressed as QUS index. The QUS index is a composite parameter derived from the two basic measurements generated by the QUS, that is, the speed of sound (SOS) and the broadband ultrasound attenuation (BUA). Research has shown the QUS index (QUI) to be a more useful determinant of bone health status capable of differentiating subjects with a history of fractures as well as predicting future fracture risk in both men and women as well as discriminating women with low BMD from healthy postmenopausal women [43–46]. In addition, several studies have also reported a strong correlation between QUI obtained from QUS and DEXA measurements [58–60]. The QUS index assesses both dimensional structure and bone strength and therefore has a range of clinical applications.

The instrument was calibrated every morning using a phantom, according to the manufacturer's recommendations to protect the long-term stability of the measurement tool.

2.6. Confounders. Potential confounders were purposefully selected using directed acyclic graphs (DAGs), in combination with discussions with content experts (Figure 1) [61]. These included demographic data (age and gender), clinical measures (BMI), lifestyle factors (smoking status, diet, and physical activity), the use of medications likely to be associated with bone metabolism or risk of falls (antiepileptics, antidepressants, and inhaled steroids), and the use of vitamin D and calcium in the form of supplements. Vitamin D and calcium are recognized regulators of bone homeostasis [62].

Data on demographic and lifestyle factors such as age, gender, and smoking habits were collected via self-report questionnaires. Data on medications including the use of vitamin D and calcium was also collected via self-report questionnaires and additionally via linkage to Medicare Australia and Pharmaceutical Benefits Scheme (PBS) that enabled collection of information on prescription drug use. BMI (weight/height^2; kg/m^2) was calculated during the clinical visit using height readings measured with a seca wall-mounted stadiometer and weight readings measured with a digital scale.

Physical activity (PA) was measured using step count. Study participants were required to wear a pedometer for seven consecutive days during waking hours to enable mean daily steps to be calculated. Additionally, nutritional assessment was carried out using the Australian Recommended Food Score (ARFS) [63]. The ARFS was calculated based on national recommendations in the Dietary Guidelines for Australian Adults and the core foods given in the Australian Guide to Healthy Eating (AGHE). Respondents are able to obtain a total of 74 points. As a result of missing data, HCS participants were only able to score a possible total Australian Recommended Food Score (TARFS) of 67 points. The scoring method is described in Table 3. A higher score is indicative of greater diet quality.

2.7. Statistics. The nature of the relationship between our selected autoantibodies and bone mass was determined using

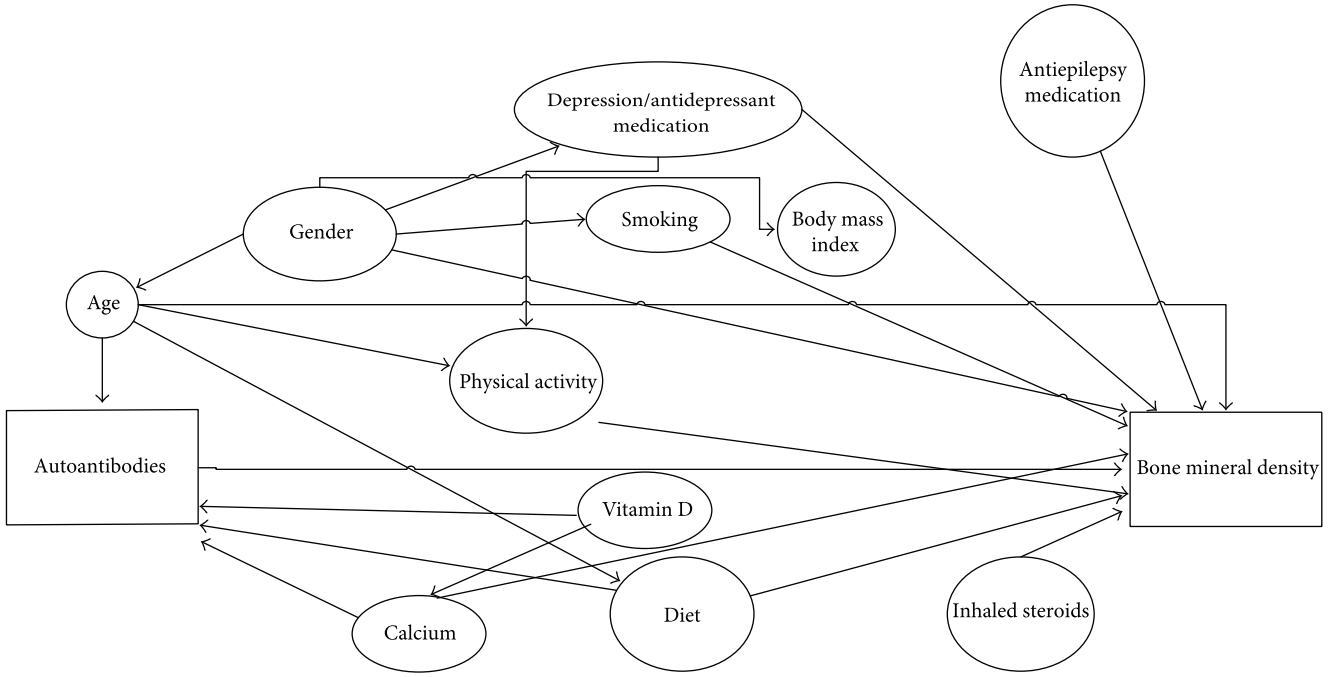

Figure 1: Directed acyclic graphs: determining confounding variables.

Table 3: Scoring method for foods listed in HCS DQESv2.

Food group	Items awarded 1 point	ARFS
Vegetables	>4 vegetables per day; potatoes cooked without fat; tomatoes fresh/canned; lettuce/endive/salad greens; carrots; cabbage/brussels sprouts; cauliflower; broccoli; celery; silver beet or spinach; peas; green beans; bean sprout or alfalfa sprout; pumpkin; onion or leeks; sweet corn; sweet potato; coleslaw; mushrooms; zucchini	20
Fruits	2 pieces of fruit/day; 1/week of each of fruit or vegetable juice; canned or frozen fruit; oranges or other citrus; apples; pears; bananas; melons (water, rock, honeydew); pineapple; strawberries; apricots; peach/nectarines; mango/pawpaw; avocado	14
Protein foods	Nuts; peanut butter or peanut paste; 1–4/week of each of beef, lamb, pork, chicken, fish (steamed, baked, or grilled fish/canned (salmon, tuna, sardines)); ≥1/week of each of baked beans, soya beans/soy beans and tofu; use up to 2 eggs per week	11
Grains	≥1/week of each of the following bread types with high fibre, wholemeal; ≥4 slices bread per day; ≥1/week All-Bran, Sultana bran, FibrePlus, Branflakes, Weetbix, VitaBrits, Weeties; rice; pasta/noodles	12
Dairy	Reduced fat or skim; 500 mL/day; cheese 1/week; ice-cream; yoghurt 1/week; use ricotta/cottage cheese; use low-fat cheese	7
Fats	Use nil/polyunsaturated/monounsaturated margarine	1
Alcohol	Drink beer/wine/spirits 1/month up to 4 days/week; or 2 glasses maximum/day	2

linear regression models. Four linear regression models were developed for each of our autoantibodies. The association between each autoimmune marker and QUS index was initially examined alone. Thereafter, we included other factors known to influence bone mineral density to each autoantibody model:

(i) Autoantibody alone (model 1)

(ii) Autoantibody alone run separately for male and female gender (model 2a and 2b)

(iii) Autoantibody + age (model 3)

(iv) Autoantibody + age + gender + smoking class + BMI + TARFS + vitamin D + calcium (model 4)

(v) Autoantibody + age + gender + smoking class + BMI + TARFS + vitamin D + calcium + physical activity + antidepressants + inhaled steroids + antiepileptics (model 5)

Given that there was no statistically significant association with either physical activity, antidepressants, inhaled steroids, or antiepileptic medication use and QUS index in univariate regression, these variables were only added in sensitivity analysis. We additionally examined the impact on

bone of expressing more than one autoantibody. All analyses were performed in Stata software v11 [64]. Results are expressed as coefficients with corresponding 95% confidence intervals and p values. Significance was set at $p < 0.05$.

3. Results

3.1. Characteristics of Study Participants and Association with Bone Mineral Density. There was a total of 2812 study participants included in this study following the exclusion of persons with clinical autoimmune disease ($n = 445$) and osteoporosis ($n = 61$). Amongst these participants in the community-dwelling sample, 1246 (44%) were male, median age was 65 years, mean total Australian Recommended Food Score (ARFS) was approximately 28/67, and median step count was 6534.25 per day. Notably, the majority of our sample were overweight ($n = 1151$; 40.93%) and had never smoked ($n = 1432$; 50.92%). Additionally, 309 (11%) and 47 (2%) study participants were noted to be using calcium and vitamin D supplements, respectively, according to self report and linkage data. Other demographic and disease-related characteristics are presented in Table 4.

Table 5 describes the coefficients for the univariate linear regression analysis (using available cases) for our selected covariates in relation to QUS index. As expected, QUS index was significantly associated with age, gender, smoking, BMI, diet (TARFS), vitamin D, and calcium intake. The results indicated that QUS index was significantly different for males compared to their female counterparts, for individuals who had never smoked compared to past and current smokers, for individuals with a BMI of overweight and obese compared to those in the normal BMI category, and for individuals who were on vitamin D and calcium supplements compared to those who were not.

Linear regression established that a 1-year increase in age elicited a 0.01 decrease in QUS index ($p = 0.006$; 95% CI: −0.02–0.004). Males demonstrated a QUS index of 0.32 units higher than females ($p = 0.000$; 95% CI: 0.20–0.44). When compared to normal BMI, being underweight resulted in a QUS index of 0.59 units lower than individuals with a normal BMI (95% CI: −1.52–0.34; $p = 0.215$); however, this association failed to reach significance. Alternatively, a BMI of "overweight" and "obese" significantly increased QUS index by 0.33 and 0.48, respectively ($p = 0.000$; 95% CI: 0.17–0.49; 95% CI: 0.31–0.65, resp.). Similarly, past smokers had an increase in QUS index by 0.13 at borderline significance ($p = 0.050$; 95% CI: 0–0.26), whilst current smokers had a significantly decreased QUS index by 0.36 ($p = 0.003$; 95% CI: −0.60 to −0.12) compared to "never smokers." Dietary intake was also positively correlated with QUS index ($r = 0.01$; 95% CI: 0.00–0.02; $p = 0.034$). Unusually, taking vitamin D and calcium supplements negatively correlated with QUS index ($r = -0.59$; 95% CI: −1.098 to −0.073; $p = 0.025$ and $r = -0.35$; 95% CI: −0.55 to −0.15; $p = 0.001$, resp.). Moreover, increasing physical activity by 1000 steps had a negligible effect on QUS index ($r = 0.00$; 95% CI: 0.00–0.00; $p = 0.285$). Also, whilst the use of antidepressants increased QUS index by 0.07, inhaled steroid use and antiepileptics decreased QUS index by 0.15 and 0.55, respectively. Neither

Table 4: Baseline demographic and disease-related characteristics of study population, total ($n = 2812$).

Descriptive characteristics	Median (IQR); n (%); mean (SD)
*Age (years)—median (IQR)	65 (55–85)
Male gender—n (%)	1246 (44.31%)
*PA (step count)—median (IQR)	6534.25 (4414.63–8572.75)
TARFS—mean (SD)	27.9627 (±8.02231)
Antidepressant—n (%)	257 (9.14%)
Inhaled steroids—n (%)	127 (4.52%)
Antiepileptics—n (%)	24 (0.85%)
Calcium—n (%)	309 (10.99%)
Vitamin D—n (%)	47 (1.67%)
(i) Smoking class "never"—n (%)	1432 (50.92%)
(ii) Smoking class "ever"—n (%)	1021 (36.31%)
(iii) Smoking class "now"—n (%)	211 (7.50%)
(a) BMI < 18.5 "underweight"—n (%)	16 (0.57%)
(b) 18.5 ≤ BMI< 25 "normal"—n (%)	548 (19.49%)
(c) 25 ≤ BMI< 30 "overweight"—n (%)	1151 (40.93%)
(d) BMI ≥ 30 "obese"—n (%)	863 (30.69%)

Categorical data is presented as frequencies and proportions in brackets. Where continuous data is not normally distributed data is presented as medians with interquartile ranges included in brackets. * indicates data that is not normally distributed. Where data is normally distributed, means and standard deviations are presented. IQR: interquartile range; BMI: body mass index; PA: physical activity; TARFS: Total Australian Recommended Food Score.

Table 5: Effect of covariates on QUS index.

Variable	Coefficient	95% confidence interval	p
Age	−0.01	−0.02; −0.004	0.006
Gender	0.32	0.20; 0.44	0.000
PA (step count)	0.00	0.00; 0.00	0.285
TARFS	0.01	0.00; 0.02	0.034
Antidepressants	0.06	−0.15; 0.27	0.582
Inhaled steroids	−0.16	−0.47; 0.15	0.304
Antiepileptics	−0.57	−1.33; 0.19	0.145
Vitamin D	−0.59	−1.098; −0.073	0.025
Calcium	−0.35	−0.55; −0.15	0.001
Smoking class "ever"	0.13	0.00; 0.26	0.050
Smoking class "never"	Ref	—	—
Smoking class "now"	−0.36	−0.60; −0.12	0.003
BMI < 18.5 "underweight"	−0.59	−1.52; 0.34	0.215
18.5 ≤ BMI < 25 "normal"	Ref	—	—
25 ≤ BMI< 30 "overweight"	0.33	0.17; 0.49	0.000
BMI ≥ 30 "obese"	0.48	0.31; 0.65	0.000

PA: physical activity; TARFS: Total Australian Recommended Food Score.

of the latter medications were significantly associated with QUS index.

3.2. Autoantibody Prevalence. Autoantibody categories (borderline, positive, and negative) were defined based on healthy samples. Autoantibody prevalence varied across our sample. ANA prevalence was highest amongst our sample with approximately 17% (316/1850) found to be positive and 36% (669/1850) borderline for this autoantibody. More female study participants (172/850; 20%) were positive for ANA than their male counterparts (144/996; 14%). In 979 sera deemed to be positive or borderline for ANA that were subsequently tested for ENA, only 3% were positive with at least one of 6 specific ENA autoantibody specificities identified. Similar to ANA, more female study participants (17/501; 3%) were positive for ENA than their male counterparts (10/477; 2%). Autoantibodies to TTG, TPO, RHF, CCP, and ACGA were observed in 6% (119/1850), 9% (160/1848), 1% (19/1660), 4% (8/188), and 12% (223/1830) of our sample, respectively. Autoantibodies to CCP, TTG, and cardiolipin were the only ones to present more prevalently amongst males when compared to their female counterparts. Anti-RHF was the least prevalent autoantibody within our sample. Autoantibody prevalence is shown in Table 6.

3.3. Correlations between Quantitative Ultrasound Index (QUS Index) and Biochemical and Clinical Variables. The details of the linear regression analyses examining the association between autoantibodies and QUS index are presented in Tables 7 and 8. ANA positivity was negatively correlated with QUS index. The latter univariate association showed borderline significance ($r = -0.20$; 95% CI: −0.40–0.00; $p = 0.046$). After adjusting for age, the latter association was observed to be approaching significance; however, statistical significance further diminished with the addition of other covariates. After adjusting for age, those who were ANA positive had an average 0.19 lower QUS index than their negative counterparts ($p = 0.058$). Notably, as we moved from borderline to positive autoantibody categories, a larger decrease in QUS index is observed.

Although not reaching statistical significance, ENA, ANCA, TPO, TTG, CCP, and ACGA autoantibody positivity showed a similar tendency towards lower QUS index in the positive autoantibody categories compared to their negative counterparts. Anti-CCP autoantibodies elicited the largest decrease in QUS index; however, these immune markers were not significantly associated with QUS index ($r = -0.43$; 95% CI: −1.51–0.65; $p = 0.437$). Notably, anti-RHF positivity was observed to elicit an increase in QUS index compared to negative counterparts in the univariate analysis ($r = 0.45$; 95% CI: −0.44–1.34; $p = 0.325$). In general, the pattern across all autoimmune markers was similar; there was a larger effect in the univariate analysis which attenuated in the more adjusted models. Moreover, there was an apparent dose-response effect on QUS index in moving from negative to borderline to positive immune marker groups. Notably, examination of R squared (R^2) illustrated that the addition of physical activity, antidepressants, inhaled steroids, and antiepileptics did not improve the model (model 5) (R^2 data not shown).

A minority of our sample was positive for more than one autoantibody (Refer to Table 9). The specificities that largely overlapped amongst the coexpression of any two autoantibodies were ANA with TTG, TPO, ACGA, and ANCA as well as TPO and TTG, ACGA and ENA, TPO and TTG, plus ANCA and ENA, TPO, TTG, and ACGA. Sensitivity analyses examining the impact on bone being positive for more than one autoantibody yielded some significant results. Individuals who were positive for both ANCA and ACGA suffered a QUS index 0.84 less than their negative counterparts (95% CI: −1.53 to −0.15; $p = 0.017$) whilst individuals who were positive for TPO and TTG suffered a QUS index 0.85 less than their negative counterparts (95% CI: −1.63 to −0.06; $p = 0.036$). Even fewer individuals were positive for more than two autoantibodies, and the coexpression of three autoantibodies did not yield any significant correlation with QUS index.

4. Discussion

Despite existing literature pointing to a potential role of autoantibodies in modulating bone mass, it remains a relatively underresearched subject matter. Notably, the majority of existing research has investigated the relationship between autoantibodies and QUS using samples with clinical autoimmune disease [14–28]. Our results failed to observe any significant association between most of our autoimmune markers and QUS index. Nonetheless, this study provides novel data towards efforts aimed at ascertaining the potential role of autoantibodies in pathogenic bone loss. The results are particularly important, as our study comprised an elderly population sample with no clinical autoimmune disease, thus eliminating the influence of a range of immunomodulatory drugs on our observed outcomes. Moreover, we found only one study investigating the association between autoantibodies and bone utilizing QUS parameters amongst a healthy population sample [65]. Additionally, our findings also contribute to data examining the association between a range of anthropometric measurements and QUS index of the calcaneus amongst an elderly sample.

Although BMD obtained by DEXA is a standard diagnostic technique for osteoporosis, it is difficult to apply in community-based studies because of a lack of portability, high costs, and exposure to ionizing radiation [66]. At present, the QUS has generated widespread interest particularly as a population screening tool as it gives a quick evaluation of bone that is reportedly highly correlated with DEXA measurement of BMD, it is inexpensive and easy to carry, and it estimates the bone density of the calcaneus whilst also providing some information concerning the structural organization of the bone [66–69]. The QUS therefore improves accessibility to testing particularly amongst patients with restricted mobility.

Our results revealed a significant association between our QUS parameter and age, gender, BMI, smoking habits, and diet as well as vitamin D and calcium intake. These observations coincide with previous reports, where BMD was

Table 6: Autoantibody prevalence amongst study participants.

Autoantibody	Negative all	Females	Males	Positive all	Females	Males	Borderline all	Females	Males
Anti-nuclear autoantibodies ($n = 1850$; 850 females and 996 males)	865 (47%)	334 (40%)	518 (52%)	316 (17%)	172 (20%)	144 (14%)	669 (36%)	334 (39%)	334 (34%)
Extractable nuclear antigen autoantibodies ($n = 979$; 501 females and 477 males)	938 (96%)	477 (95%)	460 (96%)	27 (3%)	17 (3%)	10 (2%)	14 (1%)	7 (1%)	7 (1%)
Anti-neutrophil cytoplasmic autoantibodies ($n = 1843$; 844 females and 995 males)	1406 (76%)	620 (73%)	782 (79%)	145 (8%)	88 (10%)	57 (6%)	292 (16%)	136 (16%)	156 (16%)
Anti-cardiolipin immunoglobulin G autoantibodies ($n = 1830$; 840 females and 986 males)	1607 (88%)	742 (88%)	862 (87%)	223 (12%)	98 (12%)	124 (13%)	—	—	—
Rheumatoid factor autoantibodies ($n = 1660$; 767 females and 899 males)	1641 (99%)	745 (71%)	893 (78%)	19 (1%)	12 (1%)	6 (1%)	—	—	—
Tissue transglutaminase autoantibodies ($n = 1850$; 850 females and 996 males)	1731 (94%)	801 (70%)	926 (74%)	119 (6%)	49 (4%)	70 (6%)	—	—	—
Thyroid peroxidase autoantibodies ($n = 1848$; 850 females and 994 males)	1688 (91%)	752 (66%)	933 (75%)	160 (9%)	98 (9%)	61 (5%)	—	—	—
Anti-cyclic citrullinated peptide autoantibodies ($n = 188$; 93 females and 95 males)	180 (96%)	90 (8%)	90 (7%)	8 (4%)	3 (3%)	5 (5%)	—	—	—

Data is presented as frequencies with proportions included in brackets.

Table 7: Correlation between autoantibodies and quantitative ultrasound index (QUS index): univariate analysis.

Autoantibody	Model 1 (autoantibody alone) [coefficient; (95% CI); p value]	Model 2a (autoantibody alone, females only) [coefficient; (95% CI); p value]	Model 2b (autoantibody alone, males only) [coefficient; (95% CI); p value]
ANA *borderline*	−0.11 (−0.26; 0.05) $p = 0.168$ $n = 1628$	−0.18 (−0.22; 0.18) $p = 0.857$ $n = 749$	−0.13 (−0.36; 0.10) $p = 0.267$ $n = 879$
ANA *positive*	−0.20 (−0.40; 0.00) $p = 0.046$ $n = 1628$	−0.15 (−0.40; 0.09) $p = 0.228$ $n = 749$	−0.16 (−0.47; 0.15) $p = 0.304$ $n = 879$
Anti-ENA autoantibodies *borderline*	0.55 (−0.38; 1.49) $p = 0.245$ $n = 877$	0.80 (−0.25; 1.84) $p = 0.135$ $n = 442$	0.27 (−1.38; 1.91) $p = 0.749$ $n = 435$
Anti-ENA autoantibodies *positive*	−0.39 (−0.99; 0.20) $p = 0.194$ $n = 877$	−0.21 (−0.86; 0.43) $p = 0.516$ $n = 442$	−0.59 (−1.70; 0.51) $p = 0.291$ $n = 435$
ANCA *borderline*	−0.02 (−0.21; 0.18) $p = 0.868$ $n = 1621$	−0.04 (−0.29; 0.21) $p = 0.744$ $n = 743$	0.01 (−0.27; 0.30) $p = 0.922$ $n = 878$
ANCA *positive*	−0.19 (−0.45; 0.07) $p = 0.160$ $n = 1621$	0.12 (−0.18; 0.42) $p = 0.441$ $n = 743$	−0.50 (−0.93; −0.05) $p = 0.028$ $n = 878$
Anti-TPO autoantibodies *positive*	−0.21 (−0.46; 0.04) $p = 0.100$ $n = 2114$	−0.18 (−0.47; 0.10) $p = 0.201$ $n = 1011$	−0.11 (−0.55; 0.33) $p = 0.618$ $n = 1099$
Anti-RHF autoantibodies *positive*	0.45 (−0.44; 1.34) $p = 0.325$ $n = 1947$	0.31 (−0.68; 1.30) $p = 0.536$ $n = 931$	0.76 (−0.80; 2.32) $p = 0.338$ $n = 1012$
Anti-TTG autoantibodies *positive*	−0.15 (−0.44; 0.14) $p = 0.306$ $n = 2114$	−0.02 (−0.42; 0.37) $p = 0.902$ $n = 1011$	−0.28 (−0.69; 0.14) $p = 0.192$ $n = 1099$
Anti-CCP autoantibodies *positive*	−043 (−1.51; 0.65) $p = 0.437$ $n = 2114$	−0.39 (−2.11; 1.33) $p = 0.658$ $n = 1011$	−0.61 (−2.03; 0.81) $p = 0.400$ $n = 1099$

Table 7: Continued.

Autoantibody	Model 1 (autoantibody alone) [coefficient; (95% CI); p value]	Model 2a (autoantibody alone, females only) [coefficient; (95% CI); p value]	Model 2b (autoantibody alone, males only) [coefficient; (95% CI); p value]
ACGA *positive*	−0.02	−0.10	−0.04
	(−0.23; 0.20)	(−0.38; 0.18)	(−0.27; 0.35)
	$p = 0.887$	$p = 0.480$	$p = 0.804$
	$n = 1610$	$n = 740$	$n = 870$

Data presented in bold represents significant results. Data presented in italics represents results approaching significance. 95% CI: 95% confidence interval; Ref: reference; ANA: anti-nuclear autoantibodies; Anti-ENA autoantibodies: anti-extractable nuclear antigen autoantibodies; ANCA: anti-neutrophil cytoplasmic autoantibodies; Anti-TPO: anti-thyroid peroxidase autoantibodies; Anti-RHF: anti-rheumatoid factor autoantibodies; Anti-TTG: anti-tissue transglutaminase autoantibodies; Anti-CCP: anti-cyclic citrullinated peptide autoantibodies; ACGA: anti-cardiolipin immunoglobulin G autoantibodies; BMI: body mass index; PA: physical activity; TARFS: Total Australian Recommended Food Score.

observed to be significantly associated with age, gender, BMI, and smoking habits [54, 70–72]. In fact, QUS variables have been acknowledged to decline with age similar to DEXA BMD measurements [73–75]. Our results confirmed the latter observation. Age-related bone loss is largely attributed to a rapid decline in sex hormones implicated in bone loss in varying amounts across both genders [71, 74, 76].

Gender as expected was positively associated with QUS index as you moved from female to male gender ($r = 0.32$; 95% CI: 0.20–0.44; $p = 0.000$). Gender-dependent differences in bone mass have been observed in both children and adults, with males reported to have a higher BMD than their female counterparts [77]. Similar gender-specific differences in QUS parameters have also been made evident by existing research [38, 54, 78, 79]. Gender-dependent differences in bone mass are linked to age-related decreases in sex hormones and differences in peak bone mass attained [74, 76]. Female gender is a well established risk factor for osteoporosis [80]. This is largely attributed to the role of sex hormones and sex hormone globulin that correlate with loss of BMD, fracture risk, and bone turnover [74, 76]. Additionally, gender differences in osteoporosis must be understood in the context of the physiology of bone maturation and skeletal growth as well as variations in anthropometric measures such as BMI between male and female genders [54, 80]. Briefly, males are acknowledged to achieve similar or higher bone density than females and at a later age [80]. Additionally, though gradual loss of bone mass is common across both genders with age, women tend to lose bone at a faster rate than their male counterparts [80]. Moreover, estrogen deficiency which plays a major role in osteoporosis development for both genders is noted to be more pronounced for women and begin at a younger age [74, 76, 80]. Furthermore, males are believed to have higher BMI than females and new research has also highlighted gender-related variations in molecular signaling between bone and muscle independent of purely mechanical interactions that result in gender differences in the acquisition and age-related loss in bone and muscle tissue [70, 71, 81]. Body weight is a known protective factor of bone loss [81]. As was depicted in our results, QUS index amongst individuals who are classified as overweight or obese is higher than those within the normal BMI category, $r = 0.33$; 95% CI: 0.17–0.49; $p = 0.000$ and $r = 0.48$; 95% CI: 0.31–0.65; $p = 0.000$, respectively. In this regard, a higher body weight is believed to lead to greater mechanical loading of bone with subsequent stimulation of bone formation and an increase in bone density [82]. Indeed, BMI has been reported to positively correlate with QUS index in postmenopausal women as well as in older male and female population samples [83, 84]. QUS index was lower amongst those classified as underweight when compared to normal BMI. This latter association however failed to reach statistical significance, an observation that is likely to have resulted from the small proportion of our sample that was underweight ($n = 16$).

Furthermore, our results coincide with reports of a recent meta-analysis that observed a significantly reduced bone mass amongst smokers compared with nonsmokers at all bone sites [72]. In fact, "current smokers" are observed to be a negative predictor of QUS parameters (BUA, SOS, and QUS index) amongst both men and women in previous studies [85]. Interestingly, autoantibodies have been proposed to mediate the effect of cigarette smoke on bone mass [13]. Notably, "smoking class ever" representing former smokers was significantly and positively associated with QUI in our elderly sample ($r = 0.13$; 95% CI: 0–0.26; $p = 0.05$). Previous studies using both the Sahara device and other sonometers have provided somewhat discrepant findings with both positive and negative associations reported between QUS parameters and previous smoking habits [86–90].

Diet was observed to positively correlate with QUS index amongst our elderly sample ($r = 0.01$; 95% CI: 0–0.02; $p = 0.034$). Our average TARF score (28 out of a possible 67 points) is suggestive of a diet that may not be consistent with consumption of a greater variety of foods as recommended by the Australian Dietary Guidelines [63]. Diet has been identified as an important mediator of osteoporosis risk [91]. In particular, excessive alcohol, caffeine, and tobacco use as well as low calcium and vitamin D are acknowledged to increase the risk of fragility fractures [92]. Inconsistent observations amongst studies investigating the association between diet and bone quality (mass and microarchitecture) in elderly samples have been attributed to differences in methods of measuring nutritional status (i.e., anthropometry and biochemical data versus anthropometry alone) as well as variations in study participant age [92]. Notably, a significant negative correlation was observed between calcium and vitamin D amongst our sample. Both calcium and vitamin D are recognized in existing literature as having a positive effect on

Table 8: Correlation between autoantibodies and quantitative ultrasound index (QUS index) after adjustment for potential confounders.

Autoantibody	Model 3 (autoantibody + age) [coefficient; (95% CI); p value]	Model 4 (autoantibody + age, gender, smoking class, BMI, TARFS, vitamin D, calcium) [coefficient; (95% CI); p value]	Model 5 (autoantibody + age, gender, smoking class, BMI, TARFS, vitamin D; calcium; antidepressants, inhaled steroids, antiepileptics; physical activity) [coefficient; (95% CI); p value]
ANA *borderline*	−0.10 (−0.25; 0.06) $p = 0.217$ $n = 1621$	−0.09 (−0.25; 0.07) $p = 0.264$ $n = 1467$	0.027 (−0.17; 0.22) $p = 0.784$ $n = 1013$
ANA *positive*	−0.19 (−0.39; 0.01) $p = 0.058$ $n = 1612$	−0.11 (−0.32; 0.09) $p = 0.276$ $n = 1467$	0.07 (−0.18; 0.32) $p = 0.586$ $n = 1013$
Anti-ENA autoantibodies *borderline*	0.52 (−0.41; 1.45) $p = 0.274$ $n = 867$	0.70 (−0.34; 1.73) $p = 0.186$ $n = 790$	0.75 (−0.48; 1.98) $p = 0.232$ $n = 549$
Anti-ENA autoantibodies *positive*	−0.39 (−0.98; 0.21) $p = 0.201$ $n = 867$	−0.61 (−1.26; 0.03) $p = 0.062$ $n = 790$	−0.95 (−1.79; 0.11) $p = 0.027$ $n = 549$
NCA *borderline*	−0.01 (−0.21; 0.19) $p = 0.911$ $n = 1610$	0.05 (−0.15; 0.25) $p = 0.624$ $n = 1465$	0.06 (−0.19; 0.30) $p = 0.648$ $n = 1012$
ANCA *positive*	−0.17 (−0.44; 0.07) $p = 0.148$ $n = 1610$	−0.10 (−0.38; 0.17) $p = 0.461$ $n = 1465$	0.12 (−0.23; 0.47) $p = 0.495$ $n = 1012$
Anti-TPO autoantibodies *positive*	−0.19 (−0.44; 0.07) $p = 0.148$ $n = 1804$	−0.15 (−0.41; 0.11) $p = 0.257$ $n = 1640$	0.035 (−0.31; 0.38) $p = 0.841$ $n = 1144$
Anti-RHF autoantibodies *positive*	−0.14 (−0.35; 0.79) $p = 0.214$ $n = 1637$	−0.11 (−0.34; 0.11) $p = 0.321$ $n = 1486$	−0.09 (−0.35; 0.17) $p = 0.497$ $n = 1040$
Anti-TTG autoantibodies *positive*	−0.15 (−0.44; 0.14) $p = 0.319$ $n = 1804$	−0.21 (−0.50; 0.09) $p = 0.166$ $n = 1640$	−0.23 (−0.60; 0.15) $p = 0.232$ $n = 1144$
Anti-CCP autoantibodies *positive*	−0.45 (−1.52; 0.63) $p = 0.415$ $n = 1.804$	−0.65 (−1.79; 0.49) $p = 0.264$ $n = 1640$	−1.36 (−2.76; 0.04) $p = 0.058$ $n = 1144$

Table 8: Continued.

Autoantibody	Model 3 (autoantibody + age) [coefficient; (95% CI); p value]	Model 4 (autoantibody + age, gender, smoking class, BMI, TARFS, vitamin D, calcium) [coefficient; (95% CI); p value]	Model 5 (autoantibody + age, gender, smoking class, BMI, TARFS, vitamin D; calcium; antidepressants, inhaled steroids, antiepileptics; physical activity) [coefficient; (95% CI); p value]
ACGA *positive*	−0.02	−0.05	−0.098
	(−0.24; 0.19)	(−0.27; 0.17)	(−0.35; 0.16)
	$p = 0.829$	$p = 0.674$	$p = 0.450$
	$n = 1599$	$n = 1454$	$n = 1005$

Data presented in bold represents significant results. Data presented in italics represents results approaching significance. 95% CI: 95% confidence interval; Ref: reference; ANA: anti-nuclear autoantibodies; Anti-ENA autoantibodies: anti-extractable nuclear antigen autoantibodies; ANCA: anti-neutrophil cytoplasmic autoantibodies; Anti-TPO: anti-thyroid peroxidase autoantibodies; Anti-RHF: anti-rheumatoid factor autoantibodies; Anti-TTG: anti-tissue transglutaminase autoantibodies; Anti-CCP: anti-cyclic citrullinated peptide autoantibodies; ACGA: anti-cardiolipin immunoglobulin G autoantibodies; BMI: body mass index; PA: physical activity; TARFS: Total Australian Recommended Food Score.

bone health as they are important nutrients for the development, growth, and maintenance of a healthy skeleton throughout life [93, 94]. Vitamin D and calcium are closely linked through vitamin D's regulatory role of intestinal calcium absorption [93]. Contradictory reports exist describing the relationship between vitamin D, calcium, and bone mass [93–100]. According to existing literature, high dietary calcium intake and not daily calcium supplementation have been reported to enhance bone mass [94]. Moreover, the beneficial effect of calcium on BMD is reportedly only evident in physically active groups [95, 96]. Additionally, low dietary calcium intake has been linked to increased turnover of vitamin D metabolites, an observation that is proposed to affect the subsequent relationship between vitamin D and BMD [97]. Similarly, research has failed to illustrate the effectiveness of vitamin D supplements in increasing BMD [98]. However, a longitudinal study of institutionalized women illustrated a positive effect on quantitative ultrasound of bone of supplementation with vitamin D3 and calcium [100]. Notably, supplementation with vitamin D3 and calcium in the latter study highlighted that only BUA was observed to reflect the positive effect on bone of the latter nutrients [100]. As we lacked dietary vitamin D and calcium data, we were unable to clarify the relationship between these nutrients and QUS index in our study.

Our results failed to show a significant correlation between the use of antiepileptics, antidepressants, or inhaled steroids and QUI. Notably, contrary to previous research indicating that QUS parameters at the heel respond to physical activity, our results show that physical activity had no effect on QUS index [85, 97, 101]. Physical exercise is an acknowledged and important mediator of bone biomechanics [102]. Muscle contraction produces mechanical stress that results in activation of osteoblasts with subsequent bone formation [103]. Exercise is additionally recognized to promote bone mass acquisition through direct mechanical loading effects on bone in addition to muscle contraction [103]. Clear clinical guidelines regarding the most appropriate type, intensity, and duration of activity to prevent bone loss are however lacking. The prevailing general rule regarding exercise and BMD is that exercises that include loading, weight-bearing elements, and muscular strengthening factors are considered to be most appropriate in the context of osteoporosis [103]. Yet not all types of physical activity that provide bone loading to the skeleton have been shown to produce bone mass benefits [97]. There are also activities that provide bone loading at one site of the body but not at other sites [97]. This is based on the premise that osteogenic effects of exercise are specific to the anatomical sites where the mechanical strain occurs [97]. The calcaneus that plays a central position in supporting body weight is considered the skeletal site where maximal ground reaction forces are applied with every heel strike during exercise [94]. Nonetheless, according to a recent meta-analysis that sought to mathematically consolidate research on the effects of walking interventions on BMD in men and women aged 50 years and older, walking has a significant ($p \geq 0.03$) positive effect on lumbar BMD but not femur or the calcaneus [104]. Moreover, studies that have examined the impact of physical activity on heel ultrasound are faulted for relying on historical self-report of physical activity [104]. On average, our sample walked 6534.25 steps per day. It is also possible that the amount of exercise undertaken by our study participants did not necessarily surpass the threshold necessary for modulating bone mass.

Interestingly, ANA was the most common autoimmune marker and the only autoantibody significantly associated with variability in QUS index in univariate analysis, an observation that was likely due to high power. The latter association was observed to be approaching significance after adjusting for age but disappeared following further adjustment for additional covariates. In particular, ANA was not significantly associated with QUS index when analysis was carried out separately for female and male genders ($r = -0.18$; 95% CI: −0.22–0.18; $p = 0.857$ and $r = -0.13$; 95% CI: −0.36–0.10; $p = 0.267$, resp.).

Existing literature has linked ANAs to lower BMD amongst cohorts with clinical autoimmune disease. In particular, anti-deoxyribonucleic acid (DNA) topoisomerase I autoantibodies have been noted to significantly correlate with

Table 9: Association between quantitative ultrasound index (QUS index) and the coexpression of more than one autoantibody (for available cases).

Autoantibody	Correlation coefficient	95% confidence interval	p value	N pos
ANA + ENA	−0.67	−1.57; 0.23	0.144	10/1625 (1%)
ANA + ANCA	−0.12	−0.48; 0.23	0.497	72/1628 (4%)
ANA + TPO	−0.30	−0.79; 0.19	0.227	40/1628 (2%)
ANA + RHF	1.11	−0.53; 2.75	0.183	5/1589 (7%)
ANA + TTG	−0.35	−0.99; 0.29	0.283	24/1628 (1%)
ANA + ACGA	−0.04	−0.48; 0.39	0.850	46/1626 (3%)
ANCA + ENA	−0.22	−1.50; 1.05	0.729	5/1619 (0.3%)
ANCA + TTG	0.23	−0.78; 1.23	0.655	10/1628 (1%)
ANCA + ACGA	**−0.84**	**−1.53; −0.15**	**0.017**	**18/1619 (1%)**
ANCA + TPO	−0.38	−1.07; 0.31	0.283	21/1627 (1%)
ANCA + RHF	1.81	−1.03; 4.65	0.211	2/1609 (0.1%)
TPO + TTG	**−0.85**	**−1.63; −0.06**	**0.036**	**15/1628 (1%)**
TPO + ACGA	0.17	−0.52; 0.87	0.621	21/1625 (1%)
ANA + ANCA + TPO	−0.64	−1.50; 0.21	0.141	12/1628 (1%)
ANA + TPO + ACGA	−0.37	−1.53; 0.79	0.533	7/1627 (0.4%)

BMD amongst a sample of Moroccan women with systemic sclerosis [105]. Additionally, anti-centromere autoantibodies have been identified as independent risk factors for bone damage amongst systemic sclerosis patients, whilst high anti-double-stranded DNA (dsDNA) autoantibody levels were observed to independently predict 10-year risk of incurring a hip fracture amongst SLE patients [20, 106]. ANAs represent one of the least researched immune markers in relation to pathologic bone loss. To our knowledge, this is the first study examining the association between ANAs and variability in bone mass in the absence of clinical autoimmunity. The production of ANAs is one of the major defining features of SLE, and their presence is part of the clinical diagnostic criteria [107]. Additionally, osteoporosis reportedly occurs in up to 68% of SLE sufferers [108]. The majority of studies examining the high prevalence of osteoporosis in SLE have however failed to explore the potential role of ANAs in mediating this relationship [108–111]. ANAs target a variety of nuclear antigens such as dsDNA, which are intimately involved in SLE pathogenesis [112]. Their exact mechanism of action in pathologic bone loss however remains unclear.

The direction and magnitude of the association between our autoimmune markers and QUS index were observed to be consistent. However, unlike ANA, the remaining autoantibodies did not reach statistical significance, likely due to the lower prevalence of these immune markers in our non-disease population. The dose-response effect was also reasonably consistent.

In particular, the small proportion of our sample positive for anti-ENA, anti-RHF, and anti-CCP autoantibodies significantly affected the power of our study and subsequently our ability to reliably estimate the association between the latter immune markers and QUS index, based on our results.

RHF was noted to have the lowest prevalence of all autoantibodies measured within our sample. The prevalence of these autoantibodies in the general population has been reported to increase with age [113, 114]. This increase is largely attributed to the effect of progressive senescence of immune function [114]. However, successfully ageing individuals (individuals lacking autoimmune or chronic disease) have been reported to have a prevalence of RHF which is not statistically significantly higher than a healthy young adult control group [115]. It is possible that the low RHF prevalence noted within our cohort was a result of the relatively good health of this sample.

It is important to note that previous research has illustrated a role for anti-RHF autoantibodies as enhancers of bone loss in the presence of anti-CCP autoantibodies [116]. However, a recent study, whereby radiographic progression in RA patients stratified by anti-CCP and RHF autoantibodies illustrated a more pronounced progression of structural damage associated with the presence of each autoantibody, contradicted these findings [19]. The latter study instead suggests an independent effect of RHF on bone loss in RA [19]. It has been proposed that the latter process is mediated by a proinflammatory environment resulting from activation of monocytes and macrophages through binding of RHF with low affinity Fc gamma (Fcγ) receptors found on their surface [13, 117, 118]. Furthermore, anti-CCP autoantibodies are acknowledged to bind to a diverse group of modified proteins in which arginine residues have been transformed into citrulline by peptidyl arginine deiminase [119]. However, not all their antigenic targets are implicated in modulating bone homeostasis [14, 120, 121]. At present, research has implicated citrullinated fibrinogen, enolase, and vimentin specificities in mediating bone loss via increased osteoclast resorption [14, 120, 121]. In particular, citrullinated vimentin receptors expressed on

the surface of osteoclasts and myeloid precursors are highly implicated [14, 120–122]. The latter highlights the potential for these autoantibodies to stimulate differentiation of bone-resorbing osteoclasts, as well as trigger osteoclast-driven local bone resorption. Anti-CCP autoantibodies are implicated in early bone loss during the preclinical phase of RA and have been reported to independently predict bone erosion in RA patients independent of measures of disease activity such as the disease activity score for RA(DAS28) and inflammation as measured by levels of C-reactive protein (CRP) [121, 123].

Thyroid dysfunction is acknowledged as having unfavourable effects on the musculoskeletal system [124, 125]. Individuals with hyperthyroidism, subclinical hyperthyroidism, and hypothyroidism have been repeatedly observed to exhibit an increased fracture risk [124, 125]. In this regard, the aetiology of thyroid dysfunction is multifactorial and it remains unclear which underlying mechanisms are responsible for the comorbid osteoporosis. Subsequently, inconsistencies in studies linking fracture risk to the action of thyroid hormones versus thyroid autoantibodies means the effect of thyroid dysfunction on bone pathophysiology remains unclear [125, 126]. Notably, a recent cross-sectional population-based study examining the association between calcaneal ultrasound parameters and thyroid status in middle-aged and elderly Chinese men observed high anti-TPO levels (≥200 IU/mL) to be associated with lower QUI ($p = 0.030$) [65]. We failed to observe any significant association between anti-TPO autoantibodies and QUS index.

Previous literature has suggested a role for anti-TTG autoantibodies in bone disease observed to occur alongside conditions such as celiac disease, ankylosing spondylitis, and psoriatic arthritis [127, 128]. Moreover, anti-TTG autoantibodies have previously been shown to act as a marker of low BMD as well as high fracture frequency amongst an asymptomatic celiac disease population sample [18, 22]. Our study was unable to find any evidence of a statistically significant relationship between these autoantibodies and QUS index, likely due to low power. Anti-TTG autoantibodies are implicated in pathogenic bone loss through a variety of pathways. In particular, TTG has recently been identified as a regulator of receptor activator of nuclear factor kappa beta ligand (RANKL) production as well as myeloid and mesenchymal stem cell (MSC) differentiation [32]. The latter two cell types are the major precursors for osteoclasts and osteoblasts, respectively, whilst RANKL is the key factor for maturation, proliferation, and fusion of preosteoclasts as well as osteoclast activation and survival [13]. Inactivation of TTG by anti-TTG autoantibodies may therefore carry serious implications for bone homeostasis.

Anti-TTG and anti-TPO autoantibody cooccurrence was noted to significantly and negatively correlate with QUS index amongst our elderly sample ($r = -0.85$; 95% CI: −1.63 to −0.06; $p = 0.036$). Similarly, ANCA and ACGA autoantibody coexpression was also noted to significantly decrease QUS index in our sample ($r = -0.84$; 95% CI: −1.53 to −0.15; $p = 0.017$). Anti-TTG and anti-TPO autoantibodies have been reported in celiac disease sufferers who develop thyroid dysfunction [129]. Alternatively, ANCA and ACGA have been reported to cooccur in anti-neutrophil cytoplasmic autoantibody-associated diseases, primary sclerosing cholangitis and glomerulonephritis [130–132]. Unlike TPO and TTG, there is a lack of literature linking ANCA and ACGA to pathologic bone loss. Indeed, our observations may be an incidental abnormality as very few healthy individuals when screened would be expected to be positive for ANCA and ACGA. However, we cannot disregard the potential impact these autoantibodies, when coexpressed, might have on bone. Nonetheless, the significance of the coexpression of the latter autoantibodies in relation to bone fragility required further investigation.

Our study is not without its limitations. Firstly, the use of a nonclinical population sample significantly affected the prevalence of autoantibodies and therefore our ability to detect any clinically significant effect on QUS index. Furthermore, it was assumed that a vast majority of our female sample would already have entered menopause; therefore, this data was not collected and we were subsequently unable to control for its influence on the relationship between QUS index and autoantibody positivity. Moreover, our measurement of amount of physical activity rather than loading which is acknowledged to be a more important mediator of bone health may have affected our ability to accurately delineate the relationship between physical activity and QUS index. Similarly, our failure to account for dietary calcium and vitamin D intake may have also affected our ability to correctly describe the relationship between the latter nutrients and QUS index. Furthermore, we must acknowledge the potential for bias based on our use of self-report questionnaires. Additionally, our study is based on the assessment of an elderly Caucasian sample and therefore extrapolation of our findings beyond this group should be taken with caution. Nonetheless, the study is significantly strengthened by its use of standardised methods in the assessment of study characteristics amongst our sample drawn from the general population.

5. Conclusion

Existing research has linked high autoantibody titres to bone loss observed to occur alongside a variety of autoimmune diseases as well as present amongst the elderly. However, our study findings did not support the notion that autoantibodies are causative in bone disease. As previously mentioned, the use of a nonclinical population sample significantly affected the prevalence of autoantibodies and therefore our ability to detect any clinically significant effect on QUS index. Moreover, due to the cross-sectional nature of this study, this is purely explorative research. It would therefore be premature to conclude that autoantibodies have no impact on bone mass. Investigating the health impact of autoimmunity on bone health is important as it can point to latent or clinically silent forms of osteoporosis. Serological tests for autoimmunity could then be used to identify individuals with no or atypical symptoms at a time when QUS or DEXA are unable to provide any valuable information. The

significance of autoantibodies in relation to bone health requires further investigation.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

Association of Cytotoxic T Lymphocyte Antigen-4 Gene Polymorphisms with Psoriasis Vulgaris: A Case-Control Study in Turkish Population

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Psoriasis is a common, chronic, and autoimmune skin disease in which dysregulation of immune cells, particularly T cells, is thought to play an important role in the pathogenesis. Cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed only on activated T cells is an immunoregulatory molecule and plays a role in the pathogenesis of autoimmune disorders. We aimed to determine whether CTLA-4 gene polymorphisms are associated with development and/or clinical features of psoriasis vulgaris (Pv). Genotyping of SNPs (−318C>T, +49A>G, and CT60A>G) in CTLA-4 gene was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 103 Pv patients and 102 controls. No statistically significant associations were detected in any of the investigated genetic models for the −318C>T polymorphism. The genotype distributions of +49A>G and CT60A>G were associated with Pv development. In haplotype analysis, while frequency of CAA haplotype was significantly higher in the control group, frequencies of CGG and CAG haplotype were significantly higher among the patients. However, all of CTLA-4 polymorphisms and haplotypes do not have an effect on severity and onset age of Pv. In conclusion, the +49A>G and CT60A>G polymorphisms may be risk factors for Pv development. Furthermore, CGG and CAG haplotypes may contribute to Pv development, while CAA haplotype may be protective against Pv.

1. Introduction

Psoriasis is a common inflammatory skin disease that affects approximately 125 million people globally [1]. The disease that exhibits a variable clinical presentation is characterized by lesions in the form of circular, red papules and plaques with a grey or silvery-white, dry scale. Psoriatic lesions are generally distributed symmetrically on the scalp, elbows, knees, lumbosacral area, and umbilicus [2, 3]. In addition, nail disease and/or psoriatic arthritis, which can be very painful and deforming, may develop in many patients with psoriasis [2–5]. The incidence of psoriasis in women and men is almost equal [3]. Psoriasis is associated with several comorbidities, such as Chron's disease [6, 7], cardiovascular syndrome [8, 9], metabolic syndrome [10–12], depression [13], and cancer [14, 15]. The disease leads to a serious reduction in the quality of a patient's life, because it is linked with social stigmatization, pain, discomfort, physical disability, and psychological distress [2]. Recently, psoriasis has begun to be defined as a disease spectrum or systemic disease because of abovementioned concomitant comorbidities. As a result, it requires lifelong treatment [16]. Although the molecular pathogenesis of the disease is still poorly understood, it is generally agreed that psoriasis is triggered by some environmental factors such as stress, infections, trauma, and drugs with a genetic background [17]. The common view about the molecular pathogenesis of the disease is that alterations in the complex interactions between T lymphocytes, dendritic cells, macrophages, mast cells, neutrophils, keratinocytes, cytokines, and chemokines cause psoriasis, and this wise

unbalanced immune response contributes to the psoriatic process [18, 19]. Psoriasis has four major clinical phenotypes, which are distinguished by the morphological characteristics of their lesions: (i) psoriasis vulgaris, (ii) guttate psoriasis, (iii) pustular psoriasis, and (iv) erythrodermic psoriasis [20]. The most common of these clinical phenotypes is psoriasis vulgaris, responsible for 90% of all cases, and is also known as plaque psoriasis [3]. In this phenotype, the lesions are dry, sharply demarcated, oval/circular plaques and can be localized all over the body, but eventually affecting mostly the knees, elbows, lumbosacral area, intergluteal cleft, and scalp[20, 21].

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is an important immunoregulatory molecule that plays a role in the maintenance of T cell homeostasis. In T cell-mediated immunological response, the interaction of MHC on antigen-presenting cell (APC) with CD28 on T cell is essential but not sufficient for T cell activation. However, the additional costimulatory factors and pathways are required for T cell activation [22, 23]. One of the costimulatory pathways is B7- (CD80/86) CD28 [22]. CD28 expressed on antigen-presenting cells by naive T cells binds to B7 (CD80/86) initiates the proliferation, differentiation, and cytokine production in T cells. Binding of CTLA-4 expressed by T cells to B7 presented on APC contributes to peripheral tolerance leading to the arrest of T cell cycle and termination of T cell activation [22, 24]. CTLA-4 acts as an inhibitor of autoimmunity, and the defects in the B7-CD28/CTLA-4 pathway may lower the threshold of autoreactive lymphocyte activation and which in turn may lead to the development of an autoimmune disease [25]. CTLA-4 molecule is encoded by the CTLA-4 gene (gene ID: 1493; OMIM*123890) located on chromosome 2p33 [26]. Several polymorphisms were identified in the CTLA-4 gene. The polymorphisms reducing the CTLA-4 expression or function may cause autoimmune clonal T cell proliferation and thus the development of autoimmune diseases [27]. In fact, some association studies indicated that there is an association between several CTLA-4 gene polymorphisms and various autoimmune diseases [28–48]. Recently, it has been shown that polymorphisms of many genes that are directly or indirectly related to the immune system and/or inflammation are associated with psoriasis. These include genes such as ADAM33 (a disintegrin and metalloprotease33) [49], TLR2 and TLR4 (toll-like receptor 2 and 4) [50], MCP-1 (monocyte chemoattractant protein-1) and RANTES (regulated upon activation normal T cell expressed and secreted) [51], TNFα (tumor necrosis factor alpha) [52], PON1 (paraoxonase) [53], IL-4 and IL-10 (interleukins) [54], HLA [55], VEGF (vascular endothelial growth factor) [56], and ERAP (endoplazmic reticulum aminopeptidase) [57]. However, there are a few studies establishing a possible relationship between CTLA-4 gene polymorphisms and psoriasis.

In the present study, we have conducted a research on three single-nucleotide polymorphisms (SNPs) in the CTLA-4 gene, because of its possible effects on expression level or function of the CTLA-4 molecule: −318C>T (in promoter), +49A>G (in exon-1), and CT60A>G (in exon-4). With this hypothesis, our goal was (i) to investigate whether the CTLA-4 gene polymorphisms are related to the development of Pv (psoriasis vulgaris) and (ii) to detect whether the CTLA-4 gene polymorphisms have an impact on the clinical features of *P. vulgaris* such as onset age and severity. In the literature, there are few studies which observed the relationship between CTLA-4 gene polymorphisms and Pv [58–61]. Yet, there are no previous studies revealing a relationship between CTLA-4 genes −318C>T, +49A>G, and CT60A>G SNPs and the development of Pv.

2. Subjects and Methods

2.1. Research Population. 103 unrelated Turkish Pv patients were selected for the experimental group, and 102 unrelated healthy Turkish people were selected for the control group. Psoriasis vulgaris patients (66 female/37 male; mean age ± SD: 37.83 ± 16.83) were recruited from a dermatologic clinic. The patients with other chronic and autoimmune diseases or cancer were excluded from the study. The control group (58 female/44 male; mean age ± SD: 37.23 ± 16.77) was formed with healthy individuals who did not have cancer, psoriasis, and other autoimmune diseases and did not have a family history of these diseases. The patients and control subjects were matched according to their gender and age. Severity of psoriasis was assessed with Psoriasis Area and Severity Index (PASI), ranging from 0 (no disease) to 72, with higher scores indicating the severity of disease [62]. To determine the association of CTLA-4 gene polymorphisms with the clinical features of Pv, the patients were divided into two groups according to the severity of disease (PASI < 12 group and PASI ≥ 12 group) and then assigned into two groups according to the onset of disease (early-onset group: <40 age and late-onset group: ≥40 age) (Table 1).

This study was conducted in accordance with the Declaration of Helsinki principles and was approved by the Ethics Committee of Meram Medical Faculty (number 2010/138). Informed consent was obtained from all the participants before the study.

2.2. Genotyping. Peripheral blood sample was taken from each patient and control subject collected in tubes containing EDTA and stored at −20°C before DNA isolation. Genomic DNA was extracted from the blood sample using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genotyping of the CTLA-4 gene −318C>T (rs5742909), +49A>G (rs231775), and CT60A>G (rs3087243) was carried out by the polymerase chain reaction-restriction length polymorphism (PCR-RFLP) using *Mse*II, *Bbv*I, and *Nco*I enzymes (New England BioLabs, Hitchin, UK). PCR reactions were performed with mixtures consisting of 0.2 μg genomic DNA, 5 μl ammonium buffer, 4.5 μl $MgCl_2$, 20 pmol of each primer, 5 unit Taq polymerase, and double-distilled H_2O up to final volume of 50 μl. The primers were designed according to the complete CTLA-4 gene sequence derived from NCBI Sequence Viewer (http://www.ncbi.nlm.nig.gov/). PCR was carried out with denaturation at 95°C for 5 minutes, followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C and finally 10 minutes at 72°C. The PCR products were digested using 10 U of *Mse*II,

Table 1: Characteristics of the study population.

	Patients				Control
Total number (n)	103				102
Female/male (n)	66/37				58/44
Age (mean ± SD (year))	37.83 ± 16.83				37.23 ± 16.77
Other features	With *P. vulgaris*				Healthy
	Unrelated				Unrelated
	Without cancer history				Without cancer history
	Without other autoimmune disorders and other chronic diseases				
Subgroups	According to the age of onset		According to the age of severity		
	Early < 40 age (n)	Late ≥ 40 age (n)	PASI < 12 (n)	PASI ≥ 12 (n)	
	88	15	59	44	

6 U of *Bbv*I, and 10 U of *Nco*I enzymes and then electrophoresed on 2.5% agarose gel, stained with ethidium bromide, and evaluated. The primers used for PCR, conditions for digestion, products of digestion, and genotypes determined according to the products of digestion are listed in Table 2.

2.3. Statistical Analysis. The SPSS 13.0 package programme was used for data analysis. Comparisons of the distributions of allele and genotype frequencies were performed by Pearson's chi-squared test. The deviation from the Hardy-Weinberg equilibrium was tested using chi-square analysis. To test the association between Pv and CTLA-4 polymorphisms, logistic regression analysis was performed according to five inheritance models (codominant 1, codominant 2, dominant, recessive, and log-additive). Odds ratios (OR), 95% confidence intervals (CI), and p values were determined using SNPStats (http://bioinfo.iconcologia.net/index.php?module=Snpstats) and SPSS 13.0 program. The linkage disequilibrium (LD) blocks and haplotypes were estimated using Haploview version 4.2 (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview). p values less than 0.05 were considered significant.

3. Results

3.1. Genotype Analysis and Association of SNPs with Pv. Table 3 shows the genotype and allele frequencies of CTLA-4 polymorphisms (−318C>T, +49A>G, and CT60A >G) in Pv patients and the control group. The genotype distributions of the examined SNPs were consistent with the Hardy-Weinberg equilibrium (HWE) (Table 3).

In multiple logistic regression analysis, −318C>T SNP was not associated with the development of Pv ($p > 0.05$ for all genetic models and T allele frequency). However, +49A>G and CT60A>G SNPs were associated with Pv. The disease-related risk was observed in the codominant 1 model (OR = 0.57, $p = 0.04$), dominant model (OR = 0.54, $p = 0.03$), and log-additive model (OR = 0.62 and $p = 0.03$) for +49A>G and in the codominant 2 model (OR = 0.29, $p = 0.004$) and recessive model (OR = 1.33, $p = 0.001$) for CT60. In addition, G allele (minor allele) frequencies of both +49A>G and CT60A>G SNPs were higher in the Pv patient (31% for +49A>G and 55% for CT60A>G) than in the control group (21% for +49A>G and 40% for CT60A>G) (OR = 0.59, $p = 0.02$ for +49A>G and OR = 0.54, $p = 0.002$ for CT60A>G).

3.2. Genotype Analysis and Association of SNPs with Clinical Features of Pv. Tables 4 and 5 present the genotype and allele frequencies of CTLA-4 SNPs in clinical subgroups of Pv (onset age of disease and severity of disease). None of the examined SNPs showed no association with onset age and severity of Pv (for all genetic models). The genotype and allele frequencies of examined SNPs did not differ between the early group and late group (Table 4) and PASI < 12 group and PASI ≥ 12 group (Table 5). The results indicated that −318C>T, +49A>G, and CT60A>G SNPs have no effect on the onset age and severity of Pv.

3.3. Linkage Disequilibrium and Haplotype Analysis. We estimated the linkage disequilibrium (LD) block by using Haploview version 4.2. The LD block was strongly made between −318C>T and +49A>G ($D' = 0.999$ and $r^2 = 0.043$), −318C>T and CT60A>G ($D' = 0.999$ and $r^2 = 0.141$), and +49A>G and CT60A>G ($D' = 1.000$ and $r^2 = 0.383$). In haplotype analysis which was performed to investigate the association between the haplotypes of LD block SNPs and Pv, four major haplotypes were detected which are CAA, CGG, TAG, and CAG (Table 6). The frequencies of these haplotypes were 0.532, 0.253, 0.110, and 0.103, respectively. A significantly higher frequency of CAA haplotype was found in controls (0.603) than in Pv patients (0.461, $p = 0.004$). In contrast, significant increases in the frequencies of CGG and CAG haplotypes were observed in patients (0.306 and 0.146, resp.) compared to healthy individuals in the control group (0.201 and 0.059, resp.; $p = 0.015$ and $p = 0.004$). These results suggest that while the CAA haplotype may have a protective effect on the development of Pv, the CGG and CAG haplotypes may be associated with the development of Pv. In haplotype analysis which was performed to investigate the association between the haplotypes and the clinical subgroups of Pv, three major haplotypes were detected which are AA, GG, and AG (Table 7). The frequencies of these haplotypes were 0.461, 0.306, and 0.233, respectively. Considering the onset age of Pv, the frequencies of these haplotypes

Table 2: Primers, conditions for digestion, products of digestion, and genotypes according to products of digestion.

SNP	Primers	Amplicon (bp)	RE	Temperature and duration of digestion	Products of digestion (bp) and genotypes
−318C>T	F: 5′-AAATGAATTGGACTGGATGGT-3′ R: 3′-TTACGAGAAAGGAAGCCGTG-5′	247	*Mse*II	37°C, overnight	CC: 247 CT: 20, 95, 132, 247 TT: 20, 95, 132
+49A>G	F: 5′-TTGCTCTACTTCCTGAAGACCTGAA-3′ R: 3′-AAAGTCTCACTCACCTTTGCAGAAG-5′	166	*Bbv*I	37°C, overnight	AA: 166 AG: 76, 90, 166 GG: 76, 90
CT60 A>G	F: 5′-CAC CACTATTTGGGATATACC-3′ R: 3′-AGGTCTATATTTCAGGAAGGC-5′	216	*Nco*I	37°C, overnight	AA: 20, 196 AG: 20, 196, 216 GG: 216

Table 3: Genotype and allele frequencies of CTLA-4 gene polymorphisms in Pv patients and control and the association of these polymorphisms with Pv.

SNP	Genotype/allele	Cases *n* (%)	Controls *n* (%)	HWE *p* (cases)	HWE *p* (controls)	Model	OR (95% CI)	*p*
rs5742909	CC	86 (0.83)	75 (0.74)	0.79	0.44	Codominant 1	1.84 (0.92–3.70)	0.85
(−318C>T)	CT	16 (0.16)	26 (0.25)			Codominant 2	1.08 (0.07–17.89)	0.22
	TT	1 (0.01)	1 (0.01)			Dominant	1.80 (0.91–3.56)	0.09
						Recessive	0.95 (0.06–15.67)	0.97
	C	188 (0.91)	176 (0.86)			Log-additive	1.67 (0.88–3.17)	0.11
	T	18 (0.09)	28 (0.14)			Minor allele	1.66 (0.88–3.11)	0.11
rs231775	AA	51 (0.50)	66 (0.65)	0.53	0.31	Codominant 1	0.57 (0.31–1.04)	0.04[a]
(+49A>G)	AG	41 (0.39)	30 (0.29)			Codominant 2	0.43 (0.15–1.25)	0.09
	GG	11 (0.11)	6 (0.06)			Dominant	0.54 (0.31–0.95)	0.03[a]
						Recessive	0.53 (0.19–1.50)	0.22
	A	143 (0.69)	162 (0.79)			Log-additive	0.62 (0.40–0.96)	0.03[a]
	G	63 (0.31)	42 (0.21)			Minor allele	0.59 (0.38–0.92)	0.02[a]
rs3087243	AA	25 (0.24)	36 (0.35)	0.11	0.65	Codominant 1	0.81 (0.42–1.55)	0.06
(CT60A>G)	AG	43 (0.42)	51 (0.50)			Codominant 2	0.29 (0.13–0.64)	0.004[a]
	GG	35 (0.34)	15 (0.15)			Dominant	0.58 (0.17–0.66)	0.07
						Recessive	1.33 (0.17–0.66)	0.001[a]
	A	93 (0.45)	123 (0.6)			Log-additive	1.38 (0.80–2.40)	0.25
	G	113 (0.55)	81(0.40)			Minor allele	0.54 (0.37–0.80)	0.002[a]

SNP: single-nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; OR: odds ratio; CI: confidence interval. [a]Statistically significant values ($p < 0.05$). Codominant 1: major allele homozygotes versus heterozygotes; codominant 2: major allele homozygotes versus minor allele homozygotes; dominant: major allele homozygotes versus heterozygotes + minor allele homozygotes; recessive: major allele homozygotes + heterozygotes versus minor allele homozygotes; log-additive: major allele homozygotes versus heterozygotes versus minor allele homozygotes.

did not differ between the early group and the late group ($p = 0.467$, $p = 0.434$, and $p = 0.243$, resp.). There was no significant difference between the PASI < 12 group and the PASI ≥ 12 group with respect to the frequencies of AA, GG, and AG haplotypes ($p = 0.069$, $p = 0.373$, and $p = 0.243$, resp.).

4. Discussion

Psoriasis is an inflammatory disease which is characterized by keratinocyte proliferation and activated T cell accumulation [63]. The incidence of psoriasis in women and men is almost equal [3]. However, in our study, the number of female patients (66) was significantly higher than the number of male patients (37). This situation is entirely coincidental and only results from the fact that the number of female patients who applied to the clinic during the study period was more than the number of male patients. Probably, the number of female patients and male patients would be close if the study period was extended a little longer or the number of patients could be increased.

Although its pathogenesis has not been well understood, psoriasis bears many features of a T cell-mediated autoimmune disease. It reveals a strong HLA association [64]. Since CTLA-4 regulates T cell activation and the proliferation through a negative feedback, the CTLA-4 gene is considered to be a candidate gene for T cell-mediated autoimmune disease. Hence, in this study, we aimed to investigate the

Table 4: Genotype and allele frequencies of CTLA-4 gene polymorphisms in the early-onset subgroup and late-onset subgroup and the association of these polymorphisms with onset age of Pv.

SNP	Genotype/allele	Early onset *n* (%)	Late onset *n* (%)	HWE *p* (early)	HWE *p* (late)	Model	OR (95% CI)	*p*
rs5742909	CC	74 (0.84)	12 (0.8)	0.62	0.67	Codominant 1	1.42 (0.35–5.75)	0.76
(−318C>T)	CT	13 (0.15)	3 (0.2)			Codominant 2	0.00 (NA)	
	TT	1 (0.01)	0 (0.0)			Dominant	1.32 (0.33–5.30)	0.7
						Recessive	0.00 (NA)	
	C	188 (0.91)	176 (0.86)			Log-additive	1.19 (0.33–4.30)	0.8
	T	18 (0.09)	28 (0.14)			Minor allele	1.19 (0.32–4.39)	0.11
rs231775	AA	45 (0.51)	6 (0.4)	0.49	0.98	Codominant 1	1.54 (0.48–5.01)	0.04
(+49A>G)	AG	34 (0.39)	7 (0.47)			Codominant 2	1.67 (0.29–9.62)	0.72
	GG	9 (0.1)	2 (0.13)			Dominant	1.57 (0.52–4.78)	0.42
						Recessive	1.35 (0.26–6.97)	0.73
	A	124 (0.7)	19 (0.63)			Log-additive	1.35 (0.62–2.98)	0.45
	G	52 (0.3)	11 (0.37)			Minor allele	1.38 (0.61–3.10)	0.43
rs3087243	AA	24 (0.27)	1 (0.07)	0.06	0.13	Codominant 1	2.07 (0.59–7.30)	0.45
(CT60A>G)	AG	35 (0.4)	10 (0.67)			Codominant 2	0.30 (0.03–2.89)	0.08
	GG	29 (0.33)	4 (0.27)			Dominant	1.35 (0.40–4.61)	0.62
						Recessive	0.19 (0.02–1.53)	0.06
	A	93 (0.53)	18 (0.6)			Log-additive	0.77 (0.36–1.63)	0.49
	G	83 (0.47)	12 (0.4)			Minor allele	1.34 (0.61–2.94)	0.47

SNP: single-nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; OR: odds ratio; CI: confidence interval.

Table 5: Genotype and allele frequencies of CTLA-4 gene polymorphisms in PASI < 12 and PASI ≥ 12 and the association of these polymorphisms with the severity of Pv.

SNP	Genotype/allele	PASI < 12 *n* (%)	PASI ≥ 12 *n* (%)	HWE *p* (PASI < 12)	HWE *p* (PASI ≥ 12)	Model	OR (95% CI)	*p*
rs5742909	CC	37 (0.84)	49 (0.83)	0.25	0.48	Codominant 1	1.26 (0.42–3.78)	0.39
(−318C>T)	CT	6 (0.14)	10 (0.17)			Codominant 2	0.00 (NA)	
	TT	1 (0.02)	0 (0.0)			Dominant	1.08 (0.38–3.10)	0.89
						Recessive	0.00 (NA)	
	C	188 (0.91)	176 (0.86)			Log-additive	0.93 (0.35–2.43)	0.88
	T	18 (0.09)	28 (0.14)			Minor allele	0.93 (0.35–2.45)	0.88
rs231775	AA	23 (0.52)	28 (0.47)	0.84	0.36	Codominant 1	1.05 (0.46–2.40)	0.04
(+49A>G)	AG	18 (0.41)	23(0.39)			Codominant 2	2.19 (0.52–9.22)	0.53
	GG	3 (0.07)	8 (0.14)			Dominant	1.21 (0.55–2.65)	0.63
						Recessive	2.14 (0.53–8.60)	0.26
	A	64 (0.73)	79 (0.67)			Log-additive	1.30 (0.72–2.34)	0.39
	G	24 (0.27)	39 (0.37)			Minor allele	1.32 (0.72–2.41)	0.37
rs3087243	AA	13 (0.3)	12 (0.2)	0.79	0.23	Codominant 1	0.50 (0.19–1.28)	0.08
(CT60A>G)	AG	35 (0.4)	24 (0.41)			Codominant 2	0.40 (0.14–1.18)	0.19
	GG	10 (0.23)	23 (0.39)			Dominant	0.46 (0.19–1.11)	0.08
						Recessive	0.61 (0.25–1.51)	0.28
	A	41 (0.47)	70 (0.59)			Log-additive	0.63 (0.37–1.07)	0.09
	G	47 (0.53)	48 (0.41)			Minor allele	1.67 (0.96–2.92)	0.07

SNP: single-nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; OR: odds ratio; CI: confidence interval.

possibility of an association between this candidate gene and Pv, which is defined as an autoimmune disease. In the present study, −318C>T, +49A>G, and CT60 polymorphisms were studied to evaluate their contributions to the pathogenesis of Pv, focusing on their potential effects on the activity and function of the CTLA-4 molecule. In fact, it has been

Table 6: Haplotype distribution belongs to CTLA-4 polymorphisms between Pv patients and control.

Haplotype	Frequency	Case/control ratios (frequency)	Chi-square	p
CAA	0.532	0.461, 0.603	8.274	0.004[a]
CGG	0.253	0.306, 0.201	5.959	0.015[a]
TAG	0.110	0.087, 0.132	2.12	0.145
CAG	0.103	0.146, 0.059	8.379	0.004[a]

Haplotypes were constructed in the following order: −318C>T (rs5742909)/+49A>G (rs231775)/CT60A>G (rs3087243). [a]Statistically significant values ($p < 0.05$).

suggested that −318C>T polymorphism is an effective promoter activity of the CTLA-4 gene and change transcription of CTLA-4 gene [65]. +49A>G polymorphism is located in the leader sequence which is important in the binding of the CTLA-4 molecule to B7.1 (CD80). CT60A>G polymorphism is considered to affect the alternative splicing and soluble CTLA-4 production [66].

Our data displayed no association between −318C>T SNP and the development of Pv. There were no differences in genotype and allele frequencies between the patient group and the control group. Likewise, Łuszczek et al. [60] found an association between polymorphism and Pv in their study. It has been also indicated that the association of −318C>T polymorphism with other autoimmune disorders supports our hypothesis. The association of −318C>T polymorphism with other autoimmune diseases such as spondyloarthropathy [67], pemphigus foliaceus [30], multiple sclerosis [38, 68], Behçet's disease [35], systemic lupus erythematosus [37, 69], Hashimoto's thyroiditis [41, 44], ankylosing spondylitis [40], and Graves' disease [70] supports our findings in which the researchers did not find any significant relationship between −318C>T and other diseases; however, an association between −318C>T polymorphism and other autoimmune disorders was found. The association of −318C>T polymorphism with childhood Graves' disease was reported in a Chinese population [34]. In a study on a Chinese population, a significant relationship was found between −318C>T polymorphism and rheumatoid arthritis [47, 71]. In the Italian systemic sclerosis patients, an association was found between −318C>T polymorphism and the susceptibility to develop systemic sclerosis [33].

+49A>G SNP is a CTLA-4 gene polymorphism which is probably the most widely studied and most commonly associated with autoimmune disorders and cancers. In our study, +49A>G polymorphism indicated a strong relationship with Pv in terms of minor allele frequency (OR = 0.59, 95% CI = 0.38–0.92, $p = 0.02$), codominant 1 model (OR = 1.54, 95% CI = 0.48–5.01 $p = 0.04$), dominant genetic model (OR = 0.54, 95% CI = 0.40–0.96, $p = 0.03$), and log-additive genetic model (OR = 0.62, 95% CI = 0.40–0.96, $p = 0.03$). In addition, +49A>G SNP might contribute to the risk of Pv development and G allele might be a risk factor in Pv development. This SNP causes substitution of threonine at position 17 to alanine in the CTLA-4 protein [72]. It has been postulated that this amino acid substitution may affect T cell activation by changing the posttranslational modification and ability of CTLA-4 to bind with B7.1 (CD80) [73]. Various studies have revealed that the +49G allele leads to decreased expression of CTLA-4 compared to +49A allele [27, 74]. Our findings are probably related to +49A>G SNP and may be explained by the inability of CTLA-4 to bind to B7 and/or by decreasing of CTLA-4 expression. Furthermore, decreased expression and/or broken binding with B7 in CTLA-4 may contribute to the pathogenesis of Pv by changing the T cell response. The findings of this study are inconsistent with the results of Tsunemi et al. [58], Kim et al. [59], and Łuszczek et al. [61] who evaluated the association between +49A>G polymorphism and Pv. Łuszczek et al. [61] studied on 141 Pv patients recruited from a Polish population and found that the allele and genotype distributions of +49A>G polymorphism are similar for the patients in the experimental group and healthy individuals in the control group. In the studies with Japanese [58] and Korean [59] populations, no association was reported between polymorphism and Pv. However, the results of some studies examining the relationship of +49A>G polymorphism with other autoimmune disorder, but not with Pv, are consistent with the results of our study. These studies revealed the association of +49A>G polymorphism with Graves' disease [27–29, 34, 70], rheumatoid arthritis [39], and ankylosing spondylitis [40]. On the other hand, it has been shown that there is no relation between +49A>G polymorphism and several autoimmune diseases such as rheumatoid arthritis [43, 71], Behçet's disease [35], vitiligo [75], systemic lupus erythematosus [37, 69], systemic sclerosis [33], spondyloarthropathy [67], ankylosing spondylitis [36, 40], pemphigus foliaceus [30], multiple sclerosis [38, 68], primary Sjögren syndrome [31], and ulcerative colitis [48].

In the present study, we observed a strong association between CT60A>G polymorphism and Pv in terms of codominant 2 (OR = 0.29, 95% CI = 0.13–0.64, $p = 0.004$), recessive (OR = 1.33, 95% CI = 0.17–0.60, $p = 0.001$), and minor allele frequency (OR = 0.54, 95% CI = 0.37–0.80, $p = 0.002$). Allele G appears to be a risk factor for the development of Pv. Łuszczek et al. [61] observed no difference in allele and genotype distributions of CT60A>G polymorphism between Pv patients and control subjects. This SNP is located in 3′ UTR (untranslated region) of the CTLA-4 gene and is supposed to affect the proportion of soluble isoform of CTLA-4 (sCTLA-4) to membrane-bound CTLA-4 (mCTLA-4). sCTLA-4 isoform is generated through alternative splicing of CTLA-4 mRNA. It has been previously suggested that the G allele on position +6230 (CT60G) may decrease sCTLA-4 transcript up to 50% [66]. Furthermore, we also observed higher frequencies of G allele and GG genotype in Pv patients than the control group. It is assumed G allele causes a decrease in CTLA-4 expression and deterioration of the balance between sCTLA-4/mCTLA-4 by blocking the alternative splicing of CTLA-4 mRNA. Chong et al. [34] have suggested that CT60A>G polymorphism plays a role in susceptibility to childhood Graves' disease. Kavvoura et al. [32] have discovered that polymorphism can be an important marker of genetic risk in Graves' disease and Hashimoto thyroiditis. Furthermore, it has also been suggested that

Table 7: Haplotype distribution belongs to CTLA-4 polymorphisms among different subgroups of Pv.

(a)

Haplotype	Frequency	Late/early ratios (frequency)	Chi-square	p
AA	0.461	0.472, 0.400	0.529	0.467
GG	0.306	0.295, 0.367	0.612	0.434
AG	0.233	0.233, 0.263	1.364	0.243

(b)

Haplotype	Frequency	PASI < 12/PASI ≥ 12 ratios (frequency)	Chi-square	p
AA	0.461	0.534, 0.407	3.288	0.069
GG	0.306	0.273, 0.331	0.793	0.373
AG	0.233	0.193, 0.263	1.364	0.243

Haplotypes were constructed in the following order: +49A>G (rs231775)/CT60A>G (rs3087243).

CT60A>G polymorphism leads to the susceptibility of vitiligo [75] and ankylosing spondylitis [40].

There are several reasons that could explain these controversial results among different studies: (i) studied populations have different ethnic features, (ii) studied populations have different sizes, and (iii) studied autoimmune disorders have already a multifactorial nature. In this study, −318C>T, +49A>G, and CT60A>G polymorphisms were selected because they can play a role on Pv pathogenesis by altering the promoter activity and transcription efficiency (for −318C>T), by altering T cell activation through post-translational modification (for +49A>G), and by affecting the alternative splicing and production of CTLA-4 isoforms (for CT60A>G). Although our population size was relatively small, we believe that our results will contribute to meta-analysis studies which have aimed at understanding the role of CTLA-4 on the pathogenesis of Pv.

5. Conclusions

To conclude, our data suggest that while there seems to be no correlation between −318C>T polymorphism and the development of Pv, +49A>G and CT60A>G polymorphisms may be associated with the development of Pv. In addition, our results present that none of the studied polymorphisms were related with the clinical features of Pv such as severity and onset age of disease. In performed haplotype analysis, CGG and CAG haplotypes were found to be the risk factor for the development of Pv, while CAA haplotype was found to be a protective haplotype for Pv. The haplotypes showed no association with severity and onset age of Pv. As a result, all of these findings suggest that +49A>G and CT60A>G polymorphisms of the CTLA-4 gene may play a role in the pathogenesis of Pv.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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