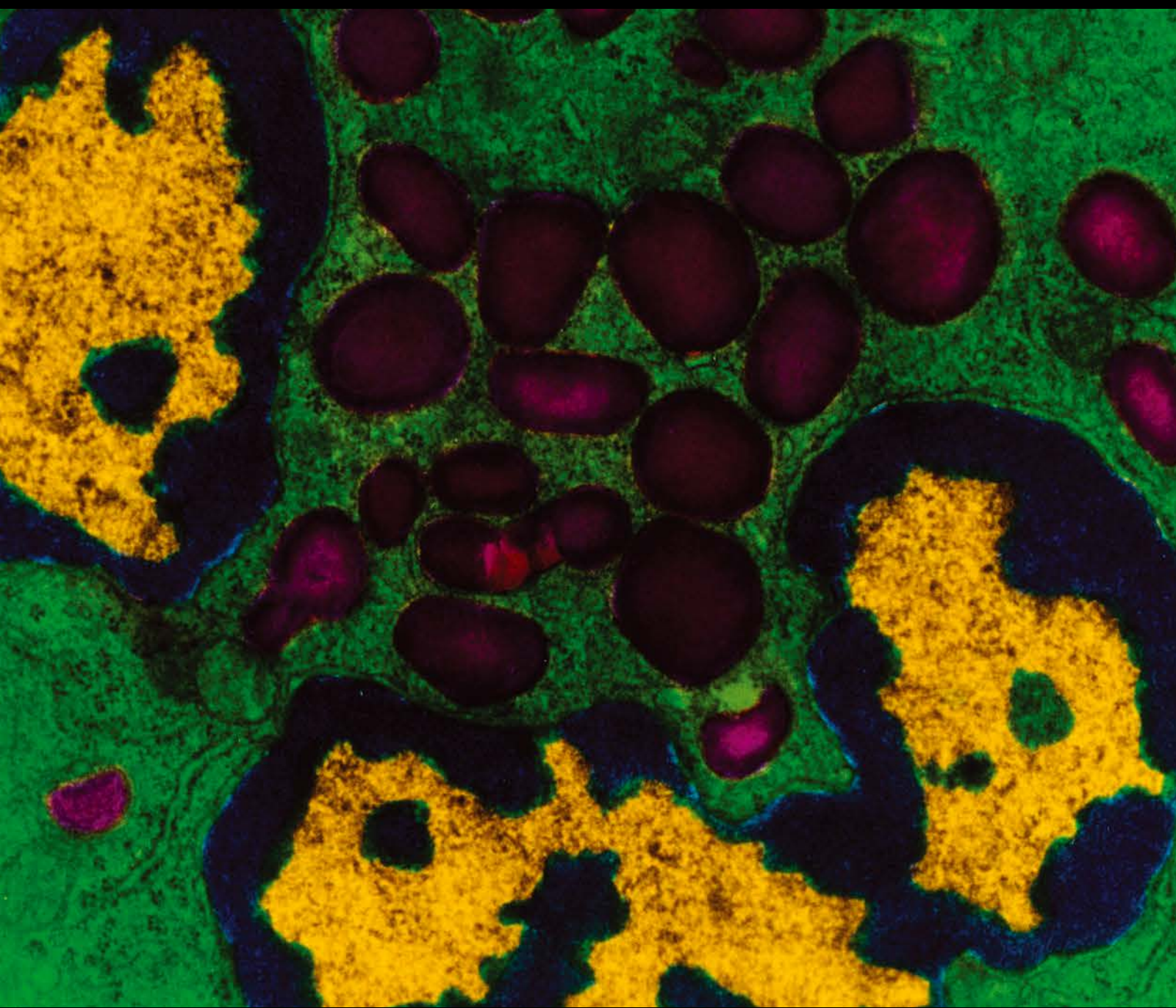
Mediators of Inflammation

Control and Resolution Mechanisms of the Inflammatory Response 2016

Guest Editors: Víctor M. Baizabal-Aguirre, Carlos Rosales, Constantino López-Macías, and Marisa I. Gómez



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Editorial Board

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Editorial

Control and Resolution Mechanisms of the Inflammatory Response 2016

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In the last 20 years the field of the innate immunity has advanced notably since the pioneering work on the discovery of Toll-like receptors. A considerable number of studies on the molecular mechanisms triggering the inflammatory response have been described. A search in PubMed with the words "*inflammatory response*" for the current year gives 11,000 articles, which means an approximate 917 articles per month and 30 per day. This amount of information reflects the interest of the scientific community not only to understand the essential mechanisms involved in the inflammatory response but also to translate this knowledge for the treatment of chronic and degenerative human diseases. Now that the main participating molecules and signaling transduction mechanisms activated during the inflammatory response have been established, researchers have begun to elucidate how a tissue is able to control/resolve inflammation and regain homeostasis. This is because a failure of the mechanisms that self-regulate and resolve the inflammatory process may lead to chronic inflammation, and this in turn may cause degenerative diseases such as cancer, diabetes, and autoimmune and cardiovascular diseases. Although many investigations have described synthetic or natural molecules that inhibit inflammation, no clear picture on the control and resolution mechanisms have emerged. This special issue is an attempt to contribute to our knowledge of the molecular programs used by the cell to control and resolve inflammation. We hope this issue may be a good reference to all interested in the complex process of the inflammation control and resolution.

Inflammation is dictated by the coordinated action of many soluble mediators and effector cells. TNF-α is a major player in this process as this cytokine is able to act on multiple cell types. In this issue, in the context of a clinically relevant entity such as asthma, J. Reyes-García et al. have explored the effects of TNF-α on tracheal smooth muscle cells and demonstrated that it inhibits L-Type Ca^{2+} channels through a ERK 1/2 pathway leading to the decrease in voltage dependent inward calcium current. They propose that this could be a compensatory mechanism during hyperresponsiveness of the airways. In the context of airway inflammation, the contribution of G. D. Albano et al. has provided evidence for the biological function of hyaluronan in regulating inflammation, using an in vitro model of nasal inflammation mediated by IL-17A. They

dissected the molecular mechanisms by which high molecular weight hyaluronan induces anti-inflammatory responses and provided evidence for the potential of this molecule as coadjuvant of the classic anti-inflammatory/antioxidative treatment of nasal epithelial cells during inflammation. In line with these studies on mucosal inflammation, C. Bernardazzi et al. have reviewed the literature about neuroimmunomodulation in the gut focusing on inflammatory bowel disease. In addition to the interplay between the nervous system and the immune system, they discussed the role of the microbiota in the regulation of the neuroimmune crosstalk involved in intestinal homeostasis and inflammation. Moving into inflammation induced during infectious diseases, M. Molteni et al. reviewed the role of Toll-Like Receptor 4 in infectious and noninfectious inflammation. They focused on the research progress during the last years about the effects of TLR4 signaling in pathological conditions.

Because inflammation has been involved in the pathogenesis of multiple diseases it is therefore important to understand the mechanisms involved in the pathogenic effects of the inflammatory responses as well as to explore different therapeutic strategies. Accordingly, G. D. Duerr et al. reported the identification of important mechanisms involved in the cardioprotective effects on metallothioneins MT1 and MT2 in a murine model of brief repetitive ischemia and reperfusion, providing valuable information that could lead to novel strategies for prevention or treatment of ischemic heart disease. E. van der Gracht et al. reviewed the current knowledge about the contribution of innate lymphoid cells in the repairing process of the epithelial barrier and tissue homeostasis and the association of these mechanisms with the control of inflammatory bowel disease. F. Capone et al. reviewed the use of the "*omics*" approach to evaluate the cytokine expression patterns in patients with cancer as a tool to define new strategies for diagnostics and prognostics. H.-P. Wu et al. analysed the IL-12 production and HLA-DR expression on cells from severe septic shock patients treated with low-dose steroids, providing important information that could help in the discussion about this treatment. V. Carregaro et al. reported the use of 15-deoxy $\Delta^{12,14}$-prostaglandin J2, to protect mice from disease aggravation in an arthritis experimental model and identify some mechanisms contributing to this process that involves several CD4 T cell populations.

Pro- and anti-inflammatory cytokines are crucial to trigger and control the inflammatory response, respectively. In this special issue M. Bigoni et al. reported the concentrations of selected pro- and anti-inflammatory cytokines in knee joints with anterior cruciate ligament injuries. In patients with this type of lesions, the reconstruction of the ligament does not decrease the incidence of posttraumatic osteoarthritis, indicating that a strong inflammatory state develops after surgery. The levels of IL-6, IL-8, and IL-10 are elevated just after knee injury and returned to almost normal levels after one month and prior to surgery, but they increased again considerably one month after reconstruction of the ligament. Thus, although the knee recovers its function, the inflammation that develops later causes damage to the bone. These findings suggest that, in addition to surgery, control of inflammation has to be considered as part of the therapy for knee injuries. Similarly, in search for new therapeutic targets for controlling inflammation, K. L. McCoy reviewed the interaction between the endocannabinoid system and the Toll-like receptor family. Because innate immune cells express cannabinoid receptors and produce endogenous cannabinoids and, in turn, the endocannabinoid system modulates local inflammatory responses, understanding their crosstalk would help fulfill the promise of cannabinoids as therapeutic agents. In line with this study, A. Janus et al. described how insulin resistance and endothelial dysfunction are connected to each other by numerous metabolic pathways and reviewed the common therapeutic targets for insulin resistance, endothelial dysfunction, and vascular inflammatory reaction at a molecular level. This helps explain the multiple effects observed with drugs currently used in management of cardiovascular disease, metabolic syndrome, and diabetes.

Toll-like receptors (TLRs) play a predominant role as pattern recognition receptors that recognize conserved molecular structures present in a wide array of microbes. The original work by Y. Qiu et al. presented evidence indicating that mice $TLR2^{-/-}$ contained a reduced number of intraepithelial type b lymphocytes (bIEL). They found that the small amount of bIEL remaining did not proliferate actively and secreted less IL-15 than normal in the intestinal mucosa. These authors concluded that lack of TLR2 is one of the factors conferring on mice a high susceptibility to dextran sulfate sodium-induced colitis. Periodontal diseases, particularly chronic periodontitis caused by *Porphyromonas gingivalis*, are one of the most common inflammatory conditions found in human beings. In the review article by P. C. Carvalho-Filho et al. the role of *P. gingivalis* in the immunopathogenesis of chronic periodontitis is clearly explained. Authors paid special emphasis on the properties of the virulence factor HmuY and the potential therapeutic strategies directed against it to reduce the inflammation caused by this pathogen. Finally, an interesting topic on the function of inflammation to restore tissue homeostasis was included as a review article by O. P. Kulkarni et al. They concisely explain the important function of different mediators of inflammation and how these mediators modify the environment to resolve inflammation. Importantly, these authors clearly point out that persistent inflammation may lead to tissue remodeling and fibrosis instead of regeneration after injury.

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Review Article

Serum Cytokinome Profile Evaluation: A Tool to Define New Diagnostic and Prognostic Markers of Cancer Using Multiplexed Bead-Based Immunoassays

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In recent years, many researchers are focusing their attention on the link between inflammation and cancer. The inflammation is involved in the tumor development and suppression, by stimulating the immune response. In particular, the transition from chronic inflammation to cancer produces angiogenic and growth factors able to repair the tissue and to promote cancer cell survival, implantation, and growth. In this contest, the cytokines contribute to the development of these processes becoming active before and during the inflammatory process and playing an important function at the various disease levels. Thus, these proteins can represent specific markers of tumor development and progression. Therefore the "cytokinome" term is used to indicate the evaluation of cytokine pattern by using an "omics" approach. Newly, specific protein chips of considerable and improved sensitivity are being developed to determine simultaneously several and different cytokines. This can be achieved by a multiplex technology that, through the use of small amounts of serum or other fluids, is used to determine the presence and the levels of underrepresented cytokines. Since this method is an accurate, sensitive, and reproducible cytokine assay, it is already used in many different studies. Thus, this review focuses on the more latest aspects related to cytokinome profile evaluation in different cancers.

1. Introduction

The most accepted model of the onset of cancer in humans shows that often, but not always, one or more genes that encode components of major signaling pathways are specifically altered in a single individual and this is the starting step driving the tumor. This event is typically repeated; thus we can have sequential mutations of these pathways along the individual's life at the transition between each tumor stage. In the final stage we often see the spread of cancer through its cancerous metastases. However, the routinely massive sequencing of genes involved in cancer has unequivocally shown that in the most common forms of human cancer we can observe that only few genes are altered in a high percentage of cancers, while the great majority of them is altered infrequently [1]. Therefore, nevertheless many scientific articles massively support the paradigm that cancer originates from mutations in specific genes, on the basis of existing data; the mechanisms for cancer diffusion through metastases must be broader than is typically thought. For example, genomic analyses of three subtypes of brain tumor show that one subtype carries a chromosomal translocation generating a new tumor-driving gene, while another subtype lacks tumor-driving mutations but possesses anomalous epigenetic modifications, and, finally, the third subtype shows neither gene mutations nor epigenetic modifications [2–4]. These observations (and others too) are confusing due to the fact that the basic biology of cancer is still a black box;

evidently more complex systemic processes are involved. Therefore, the failure of numerous drugs and treatment have only achieved increased survival but not a cure for cancer.

Over the past several years, there is accumulating evidence that chronic inflammation is involved in the development and progression of cancer [5]. The cancer cells are able to spread around a cell-to-cell information by means of a broad family of small inflammatory signaling proteins, that is, cytokines, which favor tumor growth, both facilitating genomic instability and stimulating the angiogenesis. Cytokines include chemokines, interferons, interleukins, lymphokines, and tumor necrosis factor. Cytokines are secreted by numerous cell types, including immune cells like macrophages, B lymphocytes, T lymphocytes, and mast cells, but also by endothelial cells, fibroblasts, and various stromal cells. In general, a specific cytokine may be secreted by more than one type of cell [5].

For example, macrophages are innate immune cells contributing to tumor growth and progression by their trophic role that facilitates angiogenesis, matrix breakdown, and tumor-cell motility and by promoting chronic inflammation [6]. Macrophages produce inflammatory cytokines such as TNF, IL-6, IL-12b, and IL-23 that drive inflammation through TLR signaling [7]. However, TLR signaling is insufficient to explain the strong activation of inflammatory cytokine genes in human macrophages, as previously proposed [8]. Rather, it has been found that the synergistic activation of these genes acts through interferon-γ (IFN-γ) and Toll-like receptor (TLR) signaling as important mediators of innate immunity and inflammatory disease pathogenesis [9], where IFN-γ primes macrophages for synergistic transcription of inflammatory cytokine genes upon stimulation with inflammatory factors such as TLR ligands [10]. Indeed, genome-wide analysis has shown priming of regulatory elements by IFN-γ with a synergistic induction of the transcription, so as to provide a proper chromatin environment to augment TLR-induced gene transcription. This provides transcriptional responses able to remodel the chromatin as well as support inhibition of Jak-STAT1 signaling [10].

Indeed, the inflammatory component of a developing neoplasm often includes a very differentiated leukocyte population such as neutrophils, dendritic cells, macrophages, eosinophils, and mast cells, as well as lymphocytes able to produce an assorted array of cytokines, chemotactic cytokines (or chemokines), and soluble mediators of cell killing, such as TNF-α, interleukins, growth factors, and interferons (IFNs) [11, 12]. Instead, the evolutionarily well conserved inflammatory response of higher organisms is regulated by cytokines, which are released by cells and affect the behavior of other cells, by exerting pleiotropic and redundant effects on growth promotion, differentiation, and activation, in normal cells as well as in chronic diseases such as cancer [11, 12].

Recently Mlecnik et al. (2016) proposed an immunoscore that could be used as a immunological biomarker to predict metastasis guiding the therapy [13]. However, since the genetic alterations of tumor cells did not show any relationship with the fact that the tumor develops metastasis, this should suggest that the change is a cause, rather than a consequence, of metastasis. Hence, the production of cytokines by tumor cells seems to be the molecular perturbation responsible for the development of malignant tumors [14], and this underscores the role of tumor derived cytokines.

2. Cytokinome Definition

Cytokine production and control are highly complex and multifactorial, and their effects are reflected through multiple regulatory subnetworks [11, 12]. Indeed, the cellular response to stimuli requires a perfect coordination between cellular receptors and intracellular metabolic network to integrate external stimuli and activate effective metabolic responses. The effective cellular response is in turn mediated through cytokines [11, 12]. These small informational molecules have a flow that is essentially external to the cell, covering the cell-to-cell communications; therefore, the cytokines as such do not fall in the metabolic networks that are intracellular. Their communication takes place via asynchronous modes, which is safer and energetically more economic for a multicellular system, but also independent of space and time constraints [15]. Actually, asynchronous mode means that the cell (sender) sends informational molecules without waiting for the response, but the message (cytokine) goes through a fairly homogeneous medium up to a waiting receiver, which is the receptor of another cell of the same type or even different. The receptor, which is an intermediate, receives, authenticates, and pours the message towards the inside by means of specific signaling pathways, in order to reach the true receiver which is normally the cell nucleus.

This mode, seemingly very complex, has along the way several filters that authenticate the message itself, making it sure. Furthermore, the pleiotropy ensures that the message arrives anyway. This complexity suggests that only an integral and simultaneous knowledge of the role of different cytokines may help to describe their role in the pathogenesis of cancer instead of a single cytokine assessment [16].

The "omics sciences" (genomics, transcriptomics, proteomics, fluxomics, metabolomics, etc.) are actually used to study the living organisms as a whole system. The cytokinome, according to the "omics" system of definition, can be defined as the set of all cytokines, inclusive of their mechanisms of interaction with and around cells [17]. The complexity of the cytokine system in humans can be more clearly elucidated using the cytokinome, which evaluates the complex network of interactions used to regulate either cytokine synthesis or their cognate receptors [18].

In fact, the cytokinome goes deeper in studying antagonistic and synergistic interactions among different cytokines, which can occur in many different and often redundant ways [19]. Therefore, a comprehensive understanding of cytokine functions, as well as a correct knowledge of their functional role, can be obtained only through simultaneous and coherent measurements of the cytokines concentrations in serum. This point raises the inherent difficulty of a simultaneous measurement of the cytokine

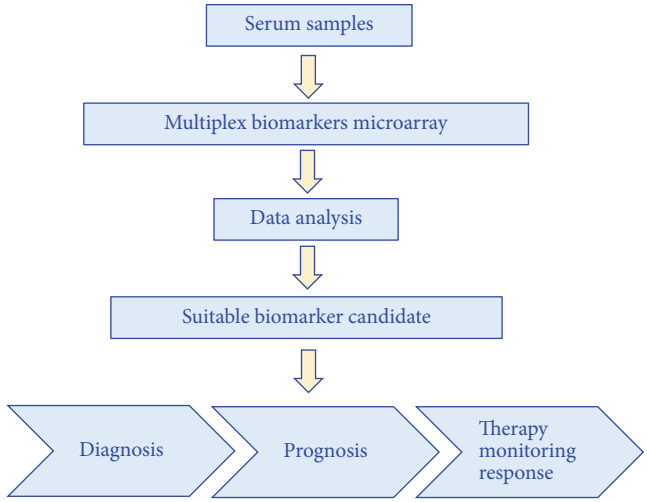

Figure 1: Flowchart related to a broad-spectrum bead-based multiplex immunoassay.

concentrations to obtain correct internal ratios among the various molecules present in the same biological fluid and also due to the often large difference in concentrations spanning several magnitude orders. At present, it is possible to effectively characterize the serum levels of cytokines using multiplexed bead-based immunoassays [18] (Figure 1).

This assay is based on the evaluation of large panels of cytokines in patient sera. The related concentrations are analyzed to identify new biomarker candidate(s), which can be used to improve the cancer diagnosis or prognosis and to evaluate the treatment response monitoring them over time.

In this review we comment on studies in which the cytokinome profile has been evaluated on sera from patients affected by several cancer types (Table 1). In this way we can evidence the importance of this approach as a tool to analyze the interaction network of cytokines both in healthy individuals and in patients affected by different cancers.

3. "Cytokinome" and Breast Cancer

Considerable advances have been made in recent years in understanding the genetics and molecular biology of breast cancer, but, for women in most western countries, this cancer remains still a major cause of death [20]. It is a heterogeneous disease and is divided into 4 main subtypes according to its clinical molecular characteristics as luminal A and luminal B and HER2 amplified and triple negative tumors. Each subtype harbors specific clinical behavior and aggressiveness, which affect disease prognosis. Luminal tumors present positivity to estrogen and/or progesterone receptors (luminal A) and can further present the amplification of the receptor of the human epidermal growth factor 2 (HER2). The latter is categorized as luminal B and is more aggressive than luminal A cancers. Some tumors present only the overexpression of HER2 and are named as HER2-amplified. Recently we have demonstrated that the positive and negative estrogen tumors show different metabolic patterns (*manuscript in press*). Finally, tumors that do not exhibit any of these receptors are classified as triple negative [21]. Breast cancer develops within a specialized tumor microenvironment that consists of numerous cell types including cancer cells, stromal cells, adipose, and infiltrating immune cells. These cells release a wide range of factors that can modulate tumor development by regulating cancer cell proliferation, survival, invasion, motility, and angiogenesis [22]. In this cancer the inflammation has an important role in tumor initiation, promotion, angiogenesis, and metastasis, and, hence, the cytokines are prominent players [23].

Depending on the staging of disease, women with breast cancer exhibit distinct patterns of circulating cytokines compared to healthy control. In the early stages, when breast cancer is localized, the patients display reduced serum levels of TNF-α and IL-12. On the other hand, patients presenting advanced disease have high systemic levels of TNF-α and IL-1β. Therefore, the cytokine profile is closely related to tumor subtype and may affect disease outcome in some instance [24]. Nicolini et al. (2006) showed that in breast cancer some cytokines such as IL-1, IL-6, IL-11, and TGF-β stimulate breast cancer proliferation and/or invasion while others such as IL-12, IL-18, and IFNs inhibit it. In particular, IFN-β has been reported to enhance estrogen and progesterone receptors [25]. Similarly, high circulating levels of some cytokines seem to be favorable prognostic indicators such as soluble IL-2R while others are unfavorable, such as IL-1b, IL-6, IL-8, IL-10, IL-18, and gp130. However, IL-2 is a potent stimulator of cellular immunity and, for this property, is the most selected interleukin for clinical trials [26]. Overall, these data underline the important role of cytokinome profile by which a global approach on assessing multiple cytokine concentrations as a measure of the interaction between the immune system and the tumor may potentially yield new methods for the diagnosis and/or prognosis of cancer patients [27].

Table 1: We report for each cancer the list of cytokines of patients with higher or lower levels compared to healthy controls, the type of cohort of patients for which the sera levels of cytokines were evaluated by multiplexed bead-based immunoassays, and related references.

Cancer	Cytokines levels	Type of cohort of patients versus controls	References
Breast cancer	↓TNF-α and IL-12	Early stage	[24]
	↑TNF-α and IL-1β	Advanced stage	[24]
	↑IL-1β, IL-6, IL-8, IL-10, and IL-18	Early stage	[26]
	↑IL-2R	Early stage	[26]
Ovarian cancer	↑VEGF, bFGF, PDGF, IL-6, IL-8, IL-1a, IL-1b, MCP-1, G-CSF, M-CSF, and TNF-α	Early stage	[31]
	↑angiogenin, angiopoietin-2, GRO, ICAM-1, IL-6, IL-6R, IL-8, IL-10, leptin, MCP-1, MIF NAP-2, osteprotegerin (OPG), RANTES, TIMP-2, and UPAR	Ascites	[32]
Lung cancer	↑sTNFRII, IL-7, TGF-α, CXCL5, CXCL9, CXCL13, CCL17, and CCL22	Cancer without other comorbidities	[33]
	↑CCL15	Early stage	[34]
	↑IL-6 and IL-8	Non-small-cell lung cancer	[35]
	↑IL-6, IL-8, IL-12, IL-17, and IFN-γ ↓IL-29 and IL-33	Non-small-cell lung cancer	[36]
Colon cancer (CRC)	↑IL-6, IL-7, IL-8, and PDGF-$\beta\beta$ ↓MCP-1	T3 or T4 rectal tumors without preoperative radiotherapy or chemoradiotherapy	[37]
	↑IL-1ra, IL-6, IL-8	Advanced stage	[37]
	↑FGF-2, TGF-α, Flt-3L, GM-CSF, INF-α2, GRO, IL-10, MCP-3, MDC, sIL-2Ra, IL-2, IL-7, IL-8, MCP-1, MIP-1β, TNF-α, and VEGF,	Metastatic CRC	[38]
Liver cancer (HCC)	↑HGF, IL-2R, s-IL-6Ra, IL-18, leptin, and sVEGFR-1 sVEGFR-2, glucagon, β-NGF, CXCL1, CXCL9, CXCL12, IL-16, PECAM-1, IFN-α, and Prolactin	HCV-related HCC	[39]
Melanoma	↑IL-1a, IL-1b, IL-6, IL-8, IL-12p40, IL-13, G-CSF, MCP-1, MIP-1α, MIP-1 β, IFN-α, TNF-α, EGF, VEGF, and TNF-RII	Patients treated with interferon-alpha2β	[40]
Gastric cancer	↑IL-6 and IL-8	Early stage	[41]
	↑IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, and IFN-γ	Distal gastric cancer	[42]
	↑IL-8, eotaxin, HGF, IP-10, MIP-1β, VEGF, Gro-a, IL-2R, IL-18, M-CSF, MIF, and MIG	Advanced stage	Our group
Pancreatic cancer	↑IL-10 and TGF-β	Primary pancreatic duct adenocarcinomas	[43]
	↑IL-5, IL-6, and IL-10	Stages II-III-IV	[44]
	↑IL-6, IL-8, IL-10, and TNF-α ↓IL-23	Newly diagnosed pancreatic adenocarcinoma	[45]
Renal cell carcinoma (RCC)	↑IL-6, IL-10, and VEGF	Metastatic RCC	[46]
	↑IL-5, IL-12, IL-6, and VEGFA	Metastatic RCC	[47]
	↑IL-6, IL-8, IL-10, G-CSF, CXCL10, CXCL11, HGF, and VEGF	RCC after undergoing nephrectomy	[48]
Thyroid cancer	↓EGF, HGF, IL-5, IL-8, and RANTES	Benign and malignant	[49]
	↑IL-6, IL-7, IL-10, and IL-13 ↓IL-8	Benign and malignant	[50]
Prostate cancer (Pca)	↑IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFN-γ, and TNF-α	Pca with cachexia	[51]
	↑IL-1β, IL-2, IL-8, IL-12, TNF-α, and IFN-γ	Pca without cachexia	[51]
	↑IL-1β and IL-12	organ-confined Pca	[51]

4. "Cytokinome" and Ovarian Cancer

The ovarian cancer is often asymptomatic and its clinical diagnosis is made when the disease is advanced and has already created metastases; for these reasons it represents the third most common gynecologic malignancy in the woman [28, 29]. Ovarian cancer has a distinctive biology and behavior at the clinical, cellular, and molecular levels and its early detection might improve clinical outcome. Inflammation is a common feature of ovarian cancer, and measurement of plasma markers of inflammation is useful for identifying candidate markers for use in screening evaluation of patients with cancer [28–30]. In serum of patients with ovarian cancer different cytokines with diagnostic value involved in the different cancer aspects were evaluated.

Many angiogenic factors are expressed and produced by tumor cells, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), VEGF, interleukins such as IL-6, IL-8, IL-1α, and IL-1β, and other cytokines, such as MCP-1, granulocyte CSF (G-CSF), M-CSF, and tumor necrosis factor-α (TNF-α). Tumor-released cytokines and their receptors expressed by endothelial and hematopoietic/lymphoid cells can induce the increase of the production also of other types of cytokines [31]. Other studies demonstrated the importance of identifying additional plasma-based proteins to predict the disease and significantly improving prediagnostic decision-making in patients with ovarian cancer, particularly the IL-6 secretion that occurs early in the pathogenesis of this cancer [52]. Also in this cancer the cytokinome profiling is of high diagnostic power and valuable tool to identify multiple soluble factors useful for its early diagnosis and therapy response assessment [32]. In particular, Matte et al. (2012) applied a multiplex cytokine array technology to measure the level of 120 cytokines in ascites from 10 ovarian cancer patients [32]. These authors demonstrated that the levels of several factors including, among others, angiogenin, angiopoietin-2, GRO, ICAM-1, IL-6, IL-6R, IL-8, IL-10, leptin, MCP-1, MIF NAP-2, osteoprotegerin (OPG), RANTES, TIMP-2, and UPAR were elevated in most malignant ascites and that IL-10 promoted the antiapoptotic activity of malignant ascites. In fact, the levels of IL-10 below 24 pg/mL in ascites correlated with survival on patients with ovarian cancer. Therefore, a better understanding of the cytokine network is essential to determine the role of ascites in ovarian cancer progression and could be more indicative of the tumor environment [32].

5. "Cytokinome" and Lung Cancer

Lung cancer results in the largest number of cancer-related deaths worldwide. More than 85% of those cases are currently classified as non-small-cell lung cancer (NSCLC), for which the predicted 5-year survival rate is 15.9% [53]. The predominant risk factor for lung cancer is smoking, accounting for approximately 90% of these lung cancer deaths. Additionally, lung cancer risk is associated with several indicators of inflammation, including pulmonary fibrosis, chronic obstructive pulmonary disease, and chronic pulmonary infections [53]. Since inflammation process is a complex response to stimuli involving the interplay of host cells and signaling molecules, such as proinflammatory and anti-inflammatory cytokines, growth and angiogenesis factors, and chemokines, the lung cancer risk has been associated with higher levels of sTNFRII, IL-7, TGF-α, CXCL5, CXCL9, CXCL13, CCL17, and CCL22 [33]. Recent evidence has shown that circulating inflammatory cytokines are associated with survival in early-stage lung cancer, and CCL15 was significantly associated with poor survival [34]. Pine et al. (2011) have focused their studies on the association between C-reactive protein (CRP), IL-6, and IL-8 and lung cancer risk [35]. Moreover, Barrera et al. (2015) showed that in patients with NSCLC there is a complex network existing between inflammatory, anti-inflammatory, angiogenic, and antiangiogenic cytokines. In particular, higher levels of IL-6, IL-8, IL-12, IL-17, and IFN-γ and lower levels of IL-33 and IL-29 are in NSCLC patients compared to healthy controls [36]. Also in lung cancer, the cytokinome profile has highlighted the opportunity to comprehensively evaluate the role of a large number of circulating inflammatory markers representative of the assortment of immune-related processes and pathways in cancer etiology and prognosis [54]. In fact, when the levels of many markers using Bio-Rad and Millipore kits were evaluated on a great number of cancer-free participants, it was possible to underline that a majority of markers were detectable in >25% of individuals on all specimen types/kits [54]. In particular, 45 Bio-Rad and 71 Millipore markers had acceptable performance and the median concentrations and intraclass correlation coefficients (ICCs) differed to a small extent across specimen types and to a large extent between Bio-Rad and Millipore [54]. Therefore, the use of this technology is reliable to perform patient serum screening.

6. "Cytokinome" and Colorectal Cancer

Colorectal cancer (CRC) is a leading cause of cancer death in developed countries [55] and cancer-related inflammation appears to be a hallmark of CRC as evidenced by the increased risk of CRC in the setting of chronic bowel inflammation [38]. Accumulating evidence suggests that some cytokines and their receptors act as regulator keys of the tumor microenvironment and are involved in many pathological entities ranging from inflammatory bowel disease to carcinogenic processes, such as the autonomous growth signaling, which influences tumor growth, invasion, and metastasis [56]. Studies have shown that CRC development is accompanied by alterations in cytokine production, which is thought to polarize from Th1 into Th2 along the colorectal adenoma-carcinoma sequence. Increased serum cytokine concentrations of IL-6, IL-7, IL-8, and PDGF, and lower levels of MCP-1 have also been reported in CRC patients compared with healthy individuals. Moreover, advanced CRCs were associated with higher levels of IL-8, IL-1ra, and IL-6 [37]. However, some other cytokines and their receptors have been found to promote tumor growth, invasion, and metastasis by acting as key regulators

of the tumor microenvironment and by being involved in many carcinogenesis-related processes, such as inflammation and autonomous growth signaling. It is therefore clinically important to elucidate the influences of multiple cytokines on the prognosis of colorectal cancer patients. For example, a recent report showed a novel cytokine-based prognostic classifier (CBPC) for prognostic prediction where 17 different circulating cytokines in metastatic CRC, such as FGF-2, TGF-α, Flt-3L, GM-CSF, INFa2, GRO, IL-10, MCP-3, MDC, sIL-2Ra, IL-2, IL-7, IL-8, MCP-1, MIP-1β, TNF-α, and VEGF, resulted in being able to predict metastatic CRC patients with a high risk of short OS [57]. Also in this cancer it has been determined that a cytokinome profile provides a simultaneous evaluation of multiple biomarkers in the same sample, and, by examining changes in multiple cytokines, it may be possible to detect more specific "cytokine footprints" for different inflammatory and neoplastic diseases. Therefore, an analysis of extensive sets of cytokines would provide more accurate information on the tumor-related immunological responses, thus bringing out the importance of individual cytokines on the immune response against cancer [58].

7. "Cytokinome" and Liver Carcinoma

Many groups are focusing their attention on the hepatocellular carcinoma (HCC) because 700,000 cases are diagnosed in each year [59]. Different factors can induce its development such as alcoholic liver disease, chronic infection with hepatitis B and hepatitis C virus, intake of aflatoxins-contaminated food, obesity, and type 2 diabetes (T2D) [60]. However, often the patients develop firstly liver fibrosis and cirrhosis and, then, HCC [61]. Our group has recently evaluated a large panel of cytokines in patients subdivided in four groups, T2D, HCV, HCV-related HCC alone, and HCV-related HCC with T2D, compared to healthy controls, to identify new markers specific for diabetes and/or HCC [39]. The obtained data evidenced that the levels of HGF, leptin, sVEGFR-1, sVEGFR-2, IL-2R, s-IL-6Ra, and IL-18, were higher in patients with T2D, HCV, and HCC. On the other hand, the levels of β-NGF, CXCL1, CXCL9, CXCL12, IL-16, and PECAM-1 increased in patients with HCV and HCC and those of IFN-α and Prolactin only in HCC patients [39].

Searching markers specific for the copresence of T2D and HCC, our results demonstrated the level increase of three interleukins (IL-2R, IL-16, and IL-18), sIL-6Ra, two chemokines (CXCL1 and CXCL12), ADIPOQ, β-NGF, HGF, and IFN-α and the decrease of leptin in T2D-HCC patients compared to those with only T2D or HCC. Moreover the comparison between the cytokine levels in T2D and T2D-HCC patients showed that CXCL9, PECAM-1, Prolactin, and glucagon levels were higher in T2D-HCC patients compared to T2D patients, and VEGFR-1 and sVEGFR-2 were lower in T2D-HCC patients [39]. Hence, also in the case of multifactorial HCC, the cytokinome analysis can be used to identify new markers useful for improving the prognosis of its progression [39].

8. "Cytokinome" and Melanoma

Melanoma is a malignant disease about which it is important to underline that if it is diagnosed in early stages, the disease is highly curable but if it is diagnosed at an advanced metastatic stage, its prognosis becomes poor [62]. However, the melanoma is an heterogeneous disease, and its pathogenesis depends on DNA mutations leading to a malignant transformation that induces an increased production of multiple growth factors and cytokines [63]. Different studies have highlighted the cytokines role in melanoma. In particular, the paper of Yurkovetsky et al. (2007) is considered the first broad multimarker screening of serum proteins for cytokines and other proinflammatory and proangiogenic proteins of patients with melanoma. This screening has shown that concentrations of 15 biomarker proteins (IL-1α, IL-1β, IL-6, IL-8, IL-12p40, IL-13, G-CSF, MCP-1, MIP-1α, MIP-1β, IFN-α, TNF-α, EGF, VEGF, and TNF-RII) were significantly higher in patients with resected high-risk melanoma compared with healthy controls. Moreover, IFN-alpha2β therapy resulted in a significant decrease of serum levels of immunosuppressive and tumor angiogenic/growth stimulatory factors (VEGF, EGF, and HGF) and increased levels of IP-10 and IFN-α [40]. Moreover, Shetty et al. (2013) evidenced that the levels of MIP-1α, IL-1Rα, IL-1β, IL-1α, IL-17, EGF, IL-12p40, VEGF, GM-CSF, and MIP-1β were significantly higher in normal controls compared to melanoma patients, while IP-10 level was lower [64].

9. "Cytokinome" and Gastric Cancer

Chronic inflammation of the gastric epithelium contributes to the pathogenesis of gastric cancer (gastric cancer) [65]. This includes a sequence of events that often begins with *Helicobacter pylori*-induced chronic superficial gastritis, progressing towards atrophic gastritis, intestinal metaplasia, dysplasia, and eventually GC [65]. Indeed *Helicobacter pylori* is etiologically involved in the development of gastric cancer and infected gastric mucosa has been shown to possess elevated levels of cytokines, for example, interleukin (IL-1β, IL-6, and IL-8) [41]. Another study reported an increase in gastric cancer risk with increased level of some cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, and IFN-γ) and evidenced that, in a population with high gastric cancer incidence and high *H. pylori* prevalence, increased circulating levels of IL-8 may indicate increased risk of gastric cancer [42]. Also our group has evaluated a large panel of cytokines characteristic of patients with gastric cancer comparing them with healthy controls. Higher amount of IL-8, eotaxin, HGF, IP-10, MIP-1b, VEGF, Gro-a, IL-2R, IL-18, M-CSF, MIF, and MIG was secreted by adenocarcinoma gastric cancer (AGC) with respect to control group [*manuscript in preparation*].

10. "Cytokinome" and Pancreatic Cancer

Pancreatic cancer is the fifth-most-common cause of cancer and its incidence is increasing at an alarming rate in western countries [66]. Unfortunately, the molecular mechanisms

underlying the development and progression of the pancreatic cancer have not yet been clarified [66]. In 1999 the first paper that reported an increase in IL-10 and TGF-β concentrations in the sera of pancreatic cancer patients and an increase in IL-4 production and a decrease in IFN-γ and IL-12 production from stimulated PBMCs was published, thus demonstrating a skewing of T-cell cytokine production towards a Th2 [43]. By multiplexing profiles of cytokines, Gabitass et al. (2011) confirmed this Th2 skewing in pancreatic cancer and extended these observations to esophageal and gastric cancer, demonstrating statistically significant increases in the plasma concentrations of the Th2 cytokines IL-5, IL-6, and IL-10 in patients when compared with controls [44]. Finally a recent study has shown higher levels of IL-6, IL-8, IL-10, and TNF-α and lower levels of IL-23 in pancreatic cancer patients compared to healthy controls [45]. This study has evidenced that these cytokines may support the blood vessels formation in the tumor microenvironment, contributing to metastatic spread by influence on the immune cells activity, inflammatory processes intensity, and the tumor cells invasiveness [45].

11. "Cytokinome" and Renal Cancer

Renal cell carcinoma (RCC) accounts for approximately 3% of adult malignancies and 90–95% of neoplasms arising from the kidney [67]. This disease is characterized by a lack of early warning signs, diverse clinical manifestations, and resistance to radiation and chemotherapy. Approximately 30% of patients with renal carcinoma (RCC) present with metastatic disease [67]. Negrier et al. (2004) evaluated the levels of IL-6, IL-10, and VEGF in patients with metastatic RCC. The results of this study showed that (i) none of these cytokines was associated with response to treatment, (ii) IL-10 was unrelated to progression-free or overall survival, and (iii) IL-6 was statistically significant in relation to progression-free survival and overall survival [46]. Moreover, a large panel of cytokines and angiogenic factors was evaluated for study if there was a correlation between the serum cytokine levels and overall survival in patients with metastatic renal cell carcinoma treated with interferon-alpha (IFN-α). This study demonstrated that serum levels of IL-5, IL-12, IL-6, and VEGFA contributed to prognostic evaluation in mRCC and to identify patients with 20% 5-year OS [47]. Also in our group we evaluated the serum levels of cytokines and chemokines in RCC patients to identify a phenotype that could be informative and prognostic in these patients. We evidenced an increase of the levels of IL-6, IL-8, IL-10, G-CSF, CXCL10, CXCL11, HGF, and VEGF in the serum of RCC patients compared to healthy controls by using a consensus of three different multiplex biometric ELISA-based immunoassays [48]. Recently, Pal et al. (2015) have assessed plasma levels of cytokines and growth factors in patients with metastatic renal cell carcinoma treated with pazopanib and subdivided into responders and nonresponders. In particular, the authors have not found significant differences between the two groups of patients in baseline whereas at 6 months of follow-up the nonresponders ended up having higher levels of HGF, IL-2R, IL-6, and IL-8, and VEGF compared to responders. This suggested that the drug resistance mechanism is at least partially related to the systemic tumor immune tolerance generation [68].

12. "Cytokinome" and Thyroid Cancer

Thyroid cancer is an uncommon type of cancer and it is usually diagnosed early. However, after the treatment this cancer may come back, also many years after treatment [69]. The most common malignant thyroid neoplasms are the well-differentiated papillary thyroid carcinomas, the follicular thyroid carcinoma, and the undifferentiated anaplastic carcinomas [69]. Data from clinical trials have shown that only few and limited positive responses were obtained with chemotherapeutic drugs in thyroid cancer. Unfortunately, the molecular bases involved in the chemotherapy-based regimens failure have not been established in most thyroid carcinomas [70]. Some studies has evidenced that several serum factors mediating inflammatory processes, angiogenesis, and tumor growth correlate with thyroid cancer development and progression. Benign and malignant thyroid disease often shows inflammatory dysregulation, which may be reflected in distinct serum cytokine profiles [71]. In 2008, Linkov et al. have evaluated by multiplex bead-based immunoassays the concentrations of 19 cytokines, chemokines, and growth factors on sera from patients with cancer, patients with benign nodular thyroid disease, and healthy subjects. They demonstrated that (i) EGF, HGF, IL-5, IL-8, and RANTES had lower levels in subjects with thyroid disease compared to normal controls and (ii) IL-8, HGF, IL-12, and MIG achieved noteworthy discrimination between benign and malignant groups [49]. In recent years it was demonstrated that malignant and benign thyroid conditions are associated with altered expression levels of interleukins, supporting the association between thyroid disease and underlying inflammatory processes. In particular, significantly higher levels of IL-6, IL-7, IL-10, and IL-13, as well as significantly lower levels of IL-8, were observed in patients with benign and malignant thyroid disease compared to controls [50].

13. "Cytokinome" and Prostate Cancer

Prostate cancer (Pca) is the most frequent cancer occurring in males, and it has been shown that its pathogenesis involves genetic as well as environmental factors [72]. Recent data suggest that inflammation may play a role in the development of prostate cancer. It has been reported that proinflammatory cytokines are capable of promoting proliferation, invasion, and angiogenesis of prostate cancer [73]. Zhang et al. (2010) have assessed a cytokines profile multiplex array in serum of patients with prostate cancer and benign prostatic hyperplasia, identifying 19 differentially expressed cytokines. Some cytokines, including IL-3, IL-6, and IL-16, ended up being upregulated in prostate cancer patients and others, including Fas/TNFRSF6, TRALR-3, and IGFBP-6, markedly downregulated [74]. On the other hand, in a study of the pathophysiology of cachexia in patients with prostate cancer,

a serum cytokinome profile of 10 biomarkers (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFN-γ, and TNF-α) was evaluated. From this profile, when compared to that of the control group, it appears that serum levels of all cytokines were significantly higher in the cachexia group, and six cytokines (IL-1β, IL-2, IL-8, IL-12, TNF-α, and IFN-γ) were significantly higher in the group with advanced Pca without cachexia. However, in the group with organ-confined Pca, only IL-1β and IL-12 levels were significantly higher compared to the control group [51]. Therefore, the cytokinome profile can be useful to identify new markers of disease progression in presence or absence of other comorbidities.

14. Cytokinome Perspectives

One of the great problems in the subclinical chronic inflammation diseases, including cancers, is discriminating which cytokines address the pathogenesis of the various diseases. This is a complex task because immune cells can release many different pathogenic cytokines, which can originate one specific disease or even many and, to complicate matters, inflammatory pathways are highly redundant [75]. This makes it very difficult to identify which specific cytokine hitting to fight a chronic disease. Often, clinically, a proinflammatory cytokine is index of disease, but not of what specific disease; it is at most a confirmation index in the presence of a recognized disease. From this derives the need to identify the whole pattern of cytokines involved in a specific inflammatory disorder, possibly together with the knowledge of the secreting immune cells, because only in this way we can identify key cells as well as the appropriate cytokines for a given inflammatory disorder. In this way we will be able to detect which cytokines might be suitable targets for fighting successfully each disease. On this issue, Schett et al. (2013) [76] share our same view [18], claiming that "human trials targeting different cytokines suggest the existence of a hierarchical framework of cytokines." This is an important observation leading to differentiation of some homogeneous sets of specific cytokines to classify in different groups the chronic inflammatory disorders which share similar pathogenic pathways in the context of resident tissue cell lineages [77].

Unfortunately, the functional redundancy of the pathways in which the cytokines are involved, also due to the structural pleiotropy of many cytokines [78], made it difficult to specifically target the key cytokines, even with antibodies [78]. This means that many prevalent chronic diseases, believed to be pathophysiologically similar and, therefore, treatable inhibiting the same target cytokine, have shown surprising failures when similarly treated [75]. From this point of view, in our opinion, one of the best available methodological approaches for this purpose is the cytokinome determination [18, 19, 39, 48], that is, the detection of the whole set of cytokines present in a biological fluid in a certain moment. Indeed, classical single cytokine assays fail to capture the physiological dynamics of the whole set of cytokines, due to their limited sensibility of response for the numerous less represented cytokines of which often we still do not have a specific assay [18, 19, 39, 48]. A valid approach to this aim is based on multiplexing measurements where the presence of less abundant cytokines can be expressed as internal ratio [18, 19, 39, 48].

In conclusion, the cytokinome approach by multiplexing measurements, although time dependent, can be very useful to identify cytokine clusters to be specifically hit, for example, by antibodies or other compounds. This methodological approach opens to a new strategy to combat what is the shared basis of almost all the major diseases of modern man, which is the subclinical chronic inflammation, thus, allowing both improving the strategies already in place and doing predictive medicine, especially when we will have a clear taxonomic classification of these diseases with clusters of cytokines linked to them.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Therapeutic Treatment of Arthritic Mice with 15-Deoxy $\Delta^{12,14}$-Prostaglandin J_2 (15d-PGJ_2) Ameliorates Disease through the Suppression of Th17 Cells and the Induction of $CD4^+CD25^-FOXP3^+$ Cells

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The prostaglandin, 15-deoxy $\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), is a lipid mediator that plays an important role in the control of chronic inflammatory disease. However, the role of prostanoid in rheumatoid arthritis (RA) is not well determined. We demonstrated the therapeutic effect of 15d-PGJ_2 in an experimental model of arthritis. Daily administration of 15d-PGJ_2 attenuated the severity of CIA, reducing the clinical score, pain, and edema. 15d-PGJ_2 treatment was associated with a marked reduction in joint levels of proinflammatory cytokines. Although the mRNA expression of ROR-γt was profoundly reduced, FOXP3 was enhanced in draining lymph node cells from 15d-PGJ_2-treated arthritic mice. The specific and polyclonal $CD4^+$ Th17 cell responses were limited during the addition of prostaglandin to cell culture. Moreover, *in vitro* 15d-PGJ_2 increased the expression of FOXP3, GITR, and CTLA-4 in the $CD4^+CD25^-$ population, suggesting the induction of Tregs on conventional T cells. Prostanoid addition to $CD4^+CD25^-$ cells selectively suppressed Th17 differentiation and promoted the enhancement of FOXP3 under polarization conditions. Thus, 15d-PGJ_2 ameliorated symptoms of collagen-induced arthritis by regulating Th17 differentiation, concomitant with the induction of Tregs, and, consequently, protected mice from diseases aggravation. Altogether, these results indicate that 15d-PGJ_2 may represent a potential therapeutic strategy in RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic disorder characterized by chronic systemic inflammation and progressive destruction of cartilage and bone. The etiology of RA is unknown, but proinflammatory cytokines play a central role in the disease development and perpetuation [1]. Among several cytokines, IL-17 is expressed in the synovial tissue of RA patients and animals models and had been implicated in the initiation and progression of arthritis [2]. In murine arthritis models, IL-17 promotes the activation of synovial fibroblasts and both leukocyte emigration and activation, resulting in the production of several inflammatory mediators and tissue lesions. For example, IL-17 has been shown to enhance joint inflammation and the tissue production of cytokines (TNF-α, IL-1β) [3], chemokines (MIP-2/CXCL2, KC/CXCL1, and IL-8/CXCL8), and matrix metalloproteinases [4]. Given the ability of IL-17 to promote RA pathology, it is plausible to suggest that pharmacologic strategies aimed at blocking or suppressing IL-17, particularly cellular Th17 function, may

deserve attention as a potential therapeutic strategy for autoimmune diseases.

The current treatments for RA are scarce and only provide symptomatic relief with limited effects on the progression of the disease. Thus, additional new therapies are needed [5]. Although Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) is a master transcriptional regulator of adipocyte differentiation, the anti-inflammatory activity of this receptor is also well described [6]. PPAR-γ modulates T cell activity by inhibiting IL-2 production in T cell receptor-stimulated Th cells [7] and by suppressing Th2 cell activity [8]. Moreover, previous studies demonstrated that PPAR-γ is an intrinsic suppressor for Th17 cell generation [9, 10]. PPAR-γ activation is thought to prevent the removal of repressor complexes from the ROR-γt gene promoter, thus suppressing ROR-γt expression and Th17 cell differentiation in an intrinsic manner. Moreover, multiple sclerosis patients are highly susceptible to PPARγ-mediated suppression of Th17 cell development, strongly asserting PPAR-γ as a promising target for specific immunointervention in autoimmune disorders [9]. Therefore, PPAR-γ ligands, including endogenous and synthetic agonists such as linoleic acid, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), and thiazolidinediones, have extensive potential in the treatment of chronic inflammatory diseases [11–13]. Therefore, we examined the potential therapeutic effect of the natural PPAR-γ agonist, 15d-PGJ_2, on collagen-induced arthritis (CIA).

2. Methods

2.1. Mice. Male DBA/1J mice weighing 18–22 g were housed at the animal facility of the Department of Pharmacology or Immunology, School of Medicine of Ribeirão Preto, University of São Paulo (Brazil), in temperature-controlled rooms (22–25°C), and received water and food ad libitum. All experiments were conducted in accordance with the National Institutes of Health (NIH) guidelines on the welfare of experimental animals and with the approval of the Ethics Committee from the School of Medicine of Ribeirão Preto.

2.2. Induction of CIA and Assessment of Arthritis. CIA was elicited in mice as previously described [14, 15]. Briefly, male DBA/1J mice (10 wk) received 200 μg bovine type II collagen (C-II) (Sigma) diluted in acetic acid and emulsified in Freund's complete adjuvant (Sigma) by intradermal (i.d.) injection at the base of the tail on day 0. Mice were boosted i.d. with collagen (200 μg diluted in acetic acid) emulsified in Freund's incomplete adjuvant (Sigma) on day 21. Mice were monitored daily for signs of arthritis as described [14, 15]. Scores were assigned based on erythema, swelling, or loss of function present in each paw on a scale of 0–3, resulting in a maximum score of 12 per mouse. When mice reached a score of 1 for clinical arthritis, they were treated with 15d-PGJ_2 (1 mg/kg) by the s.c. route daily for 7 days. Control mice received the same volume of PBS. Scoring was conducted in a blinded fashion. Mechanical hypernociception (pain) evaluation in the tibia-tarsal joint was performed with an electronic anesthesiometer (model 1601C, Life Science Instruments, California, USA) consisting of a pressure transducer connected to a digital counter force in grams (g). Capture is achieved by pressure contact of the paw pressure transducer, which is accomplished through a polypropylene tip area that is connected to the transducer. The assessment of nociception in the tibia-tarsal joint consists of the application of increasing pressure on the paw of the mouse through mesh until the animal flexes the femur and tibia, producing a paw withdrawal response. The intensity of mechanical hypernociception of the joint is measured by the absolute values of the mechanical threshold (in grams). For verification of edema, paw thickness was measured daily using a caliper, and the values are expressed in millimeters (mm). For histologic assessment, mice were euthanized 35 days after challenge, and the hind limbs were removed and demineralized thoroughly in 10% EDTA for 1-2 wk. The decalcified tissues were trimmed, dehydrated in graded ethanol, and embedded in paraffin. Serial sections (5 μm) were cut and mounted on glass slides precoated with 0.1% poly-L-lysine (Sigma). Histologic assessment was performed following routine hematoxylin and eosin staining (H&E). Ankle and joint sections were prepared and stained with H&E to study the inflammatory cell influx or using safranin-O to determine proteoglycan depletion and cartilage destruction. To measure cytokine concentrations in the inflammatory site, articular tissues were harvested, weighed, and titered in 1 mL of PBS containing complete protease inhibitor cocktail (Roche) by a tissue trimmer. Articular homogenates were centrifuged, and the supernatants were collected and stored at −70°C for determination of IFN-γ, IL-12, IL-17, and TNF-α levels by ELISA (BD Biosciences), according to the manufacturer's instructions.

2.3. T Cell Proliferation. To assess the influence of 15d-PGJ_2 treatment on T cell proliferation, popliteal and inguinal lymph nodes cells harvested from arthritic mice were removed and washed twice with PBS. Tissues were minced, and the cells were filtered through a cell strainer, centrifuged at 500 ×g at 4°C for 10 min, and resuspended in RPMI-1640 medium at 2.5×10^6 cells/mL. In some wells, cells were incubated with 15d-PGJ_2 (5 μM) or vehicle (DMSO 0.5%) 1 hour before stimulation. In all of the experiments, C-II (5 μg/mL), plate-bound anti-CD3 mAb (5 μg/mL), or medium was added to the culture and incubated for 96 h in a total volume of 200 μL per condition. Supernatants were harvested for determination of IL-17 production using ELISA, and cell proliferation was measured by overnight [^{3}H]thymidine incorporation.

2.4. Flow Cytometry. Popliteal and inguinal lymph nodes from arthritic mice were harvested 7 days after arthritis symptoms and processed, and cells were cultured with 15d-PGJ_2 (5 μM) or vehicle (PBS DMSO 0.05%) 1 hour before anti-CD3 mAb stimulation. Cells were incubated with fluorochrome-conjugated mAb anti-CD4, anti-CD25, CTLA-4, and GITR for 30 min at 4°C, washed, and fixed with BD Cytofix (BD Biosciences). Cells were permeabilized using PBS containing 1% FCS, 0.01% sodium azide, and 0.05% saponin and stained

with anti-mouse FOXP3 (all antibodies from BD Biosciences), acquired on FACS Canto II (BD Biosciences), and anayzed using FlowJo software (Tree Star).

2.5. Generation of Th17 Cells and Regulatory T Cells. $CD4^+CD25^-$ or $CD4^+CD25^+$ cells from the spleen were isolated using a $CD4^+CD25^+$ regulatory T cell kit (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instructions, and a purity of ~95% was obtained for each T cell subset. For Th17 differentiation, $CD4^+CD25^-$ cells (5.0×10^5 cells/well) were stimulated with plate-bound anti-CD3 mAb (5 μg/mL), anti-CD28 mAb (1.0 μg/mL), rmTGF-β (2.5 ηg/mL), IL-6 (10 ηg/mL), and anti-IFN-γ mAb (10 μg/mL). For Treg differentiation, $CD4^+CD25^-$ cells (5.0×10^5 cells/well) were stimulated with rmTGF-β (5 ng/mL), rmIL-2 (100 U/mL), anti-IFN-γ (10 μg/mL), and anti-IL-4 (10 μg/mL). In all experiments, 15d-PGJ_2 (5 μM) or medium was added to the culture on days 0, 3, and 5, and the cells were incubated at 37°C in 5% CO_2 for 7 days in a total volume of 200 μL per condition. As a differentiation control, nTreg ($CD4^+CD25^+$) or Th0 ($CD4^+CD25^-$) cells were cultured in the presence of IL-2 (100 U/mL) for T cell maintenance. The lymphocytes were then washed and phenotyped for the expression of surface markers using monoclonal antibodies specific for CD4 or CD3 conjugated to FITC or PerCP (BD & Biosciences eBioscience, San Diego, CA, USA). For intracellular IL-17 or FOXP3, stained cells were washed twice with PBS and centrifuged at 400 ×g for 10 minutes, followed by incubation with Cytofix/Cytoperm (BD Biosciences) for 15 minutes. Samples were again washed and incubated with a specific antibody for IL-17 conjugated to PE diluted in 1x Permwash for 10 min and after further washing in PBS were acquired on a FACSCanto II unit (BD Biosciences). Analyses were performed using FlowJo software (TreeStar, Ashland, OR, USA).

2.6. Quantitative RT-PCR. Total RNA was extracted from draining lymph nodes (inguinal and popliteal) of naïve or arthritic animals treated with 15d-PGJ_2 using RNAspin Mini Isolation Kit (GE Healthcare, Buckinghamshire, Germany) following the manufacturer's recommendations. Gene expression was normalized to the expression of the GAPDH gene: GAPDH forward: 5′-TGCAGTGGCAAAGTGGAGAT-3′; reverse: 5′-CGTGAGTGGAGTCATACTGGAA-3′; PPAR-γ forward 5′-TGAGATCATCTACACGATGCT-3′; reverse: 5′-GGAACTCCCTGGTCATGAA-3′; ROR-γt forward 5′-GCTTCCCAATGGACACTTGCAAG-3′; and reverse: 5′-AGGACAGCACACAGCTGGCAGTGG-3′; FOXP3 forward: 5′-ACAACCTGAGCCTGCACAAGT-3′; reverse: 5′-GCCCACCTTTTCTTGGTTTTG-3′.

2.7. Statistical Analysis. Data are expressed as the mean ± SEM and are representative of 2–4 independent experiments. The results of individual experiments were not combined, as they were analyzed individually. The means from different groups were compared by analysis of variance (ANOVA) followed by Tukey's test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Therapeutic Effect of 15d-PGJ_2 on the Development of Experimental Rheumatoid Arthritis. PPAR-γ is a potent modulator of inflammatory responses [16, 17]. We investigated whether PPAR-γ is expressed during collagen-induced arthritis (CIA), a murine model that shares similarities with rheumatoid arthritis (RA). CIA was elicited in DBA/1J mice, as described in Section 2, and draining lymph nodes (inguinal and popliteal) from naïve or arthritic animals were harvested 7 days after disease manifestation. As shown in Figure 1(a), the PPAR-γ mRNA transcript was highly expressed in the lymph nodes of arthritic animals when compared with the control group (naive animals). Next, the potential therapeutic effect of the PPAR-γ agonist, 15d-PGJ_2, on CIA was evaluated. Mice were treated daily with 15d-PGJ_2 (1 mg/kg) by the subcutaneous route for 7 days from the first day of clinical manifestation of disease. Controls received vehicle (PBS). As expected, control mice (vehicle-treated) developed a severe disease from day 22 until day 30 after CIA induction, exhibiting high clinical scores (Figure 1(b)), mechanical hypernociception (Figure 1(d)), and edema (Figure 1(e)) (2.43 ± 0.12). However, the treatment of arthritic mice with 15d-PGJ_2 attenuated the severity of the disease, with a reduction in the clinical scores (Figure 1(b)), mechanical hypernociception (Figure 1(d)), and swelling (Figure 1(e)). With respect to the numbers of affected paws, no significant difference was observed between the groups (15d-PGJ_2 and vehicle) (Figure 1(c)), suggesting that prostanoid treatment interfered with progression but did not prevent disease onset (see Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/9626427).

Histologic analyses of the knees at the end of the monitoring period revealed that untreated arthritic mice exhibited severe cellular infiltration (Figures 2(a) and 2(b)) and marked reductions in matrix proteoglycan (Figure 2(c)), suggesting joint cartilage damage. In contrast, these pathologic events were reduced in 15d-PGJ_2-treated animals (Figure 2(b)). Altogether, these data suggest that 15d-PGJ_2 attenuated the severity of CIA and prevented the progression of articular damage.

3.2. 15d-PGJ_2 Treatment Reduces Proinflammatory Cytokine Production. Given that the onset and progression of autoimmune diseases (including rheumatoid arthritis (RA)) are mediated by proinflammatory cytokines released into the inflammatory site, we investigated the effect of 15d-PGJ_2 treatment upon the production of TNF-α, IFN-γ, IL-17, and IL-12 in affected ankle joints. Paw samples from arthritic mice treated with vehicle (PBS) contained significantly higher concentrations of all abovementioned inflammatory cytokines compared with those of naïve mice (Figure 3). However, mice treated with 15d-PGJ_2 exhibited a significant reduction in the levels of IL-12 (Figure 3(a)), TNF-α (Figure 3(b)), IL-17 (Figure 3(c)), and IFN-γ (Figure 3(d)) compared with vehicle-treated arthritic mice.

3.3. 15d-PGJ_2 Suppresses the Inflammatory Response. Given that 15d-PGJ_2 attenuated the severity of arthritis (Figure 1),

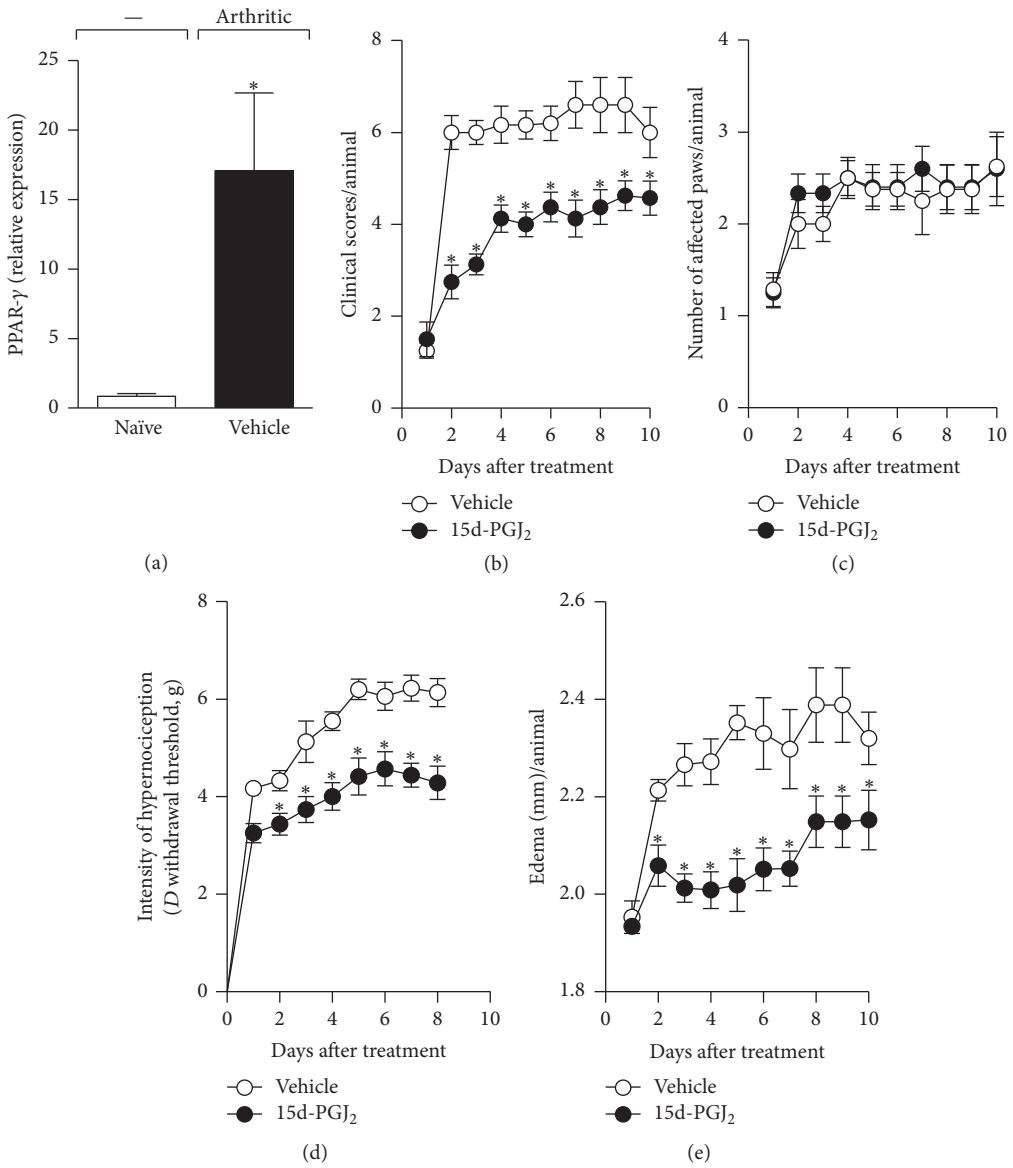

Figure 1: 15d-PGJ_2 attenuated collagen-induced arthritis. PPAR-γ mRNA expression was quantified by real-time PCR in draining lymph nodes from naïve (white bar) or collagen-immunized and challenged DBA/1 (black bar) mice on the seventh day of disease (a). Arthritic mice were treated by the subcutaneous route with vehicle (○) or 15d-PGJ_2 (1 mg/Kg) (●) for 7 d. Mice were monitored for disease progression as indicated by clinical scores (b), number of affected paws (c), hypernociception (d), and edema (e). Results are presented as the mean ± SEM, $N = 8$–10; $^*P < 0.05$ compared with the PBS-treated group.

we investigated whether treatment with this prostanoid interferes with the pattern of the Th17 response. First, we analyzed the mRNA levels of the common Th17 transcription factor, ROR-γt. RT-PCR analyses revealed that the expression of ROR-γt was increased in arthritic animals. Interestingly, the transcript for ROR-γt was decreased when arthritic animals were treated with 15d-PGJ_2 (Figure 4(a)). To investigate the impact of 15d-PGJ_2 treatment upon the collagen-specific response, draining lymph node $CD4^+$ T cells from arthritic mice were sorted and treated for 1 hour with prostanoid (5 μM). The proliferative response and IL-17 levels were measured in the culture. Using different culture conditions with specific antigen (collagen) or soluble anti-CD3 antibody for 96 hours, higher levels of IL-17 were detected relative to the medium alone. However, the addition of 15d-PGJ_2 ablated IL-17 production even under polyclonal stimulation (Figure 4(b)). Prostanoid treatment also suppressed the proliferative immune response induced by the specific (Figure 4(c)) or polyclonal (Figure 4(d)) stimuli relative to vehicle. These data demonstrate the immunosuppressive effect of 15d-PGJ_2 in the inflammatory immune response during CIA.

3.4. 15d-PGJ_2 Promotes the Treg Profile among Effector T Cells. To further explore the immunomodulatory effect of 15d-PGJ_2

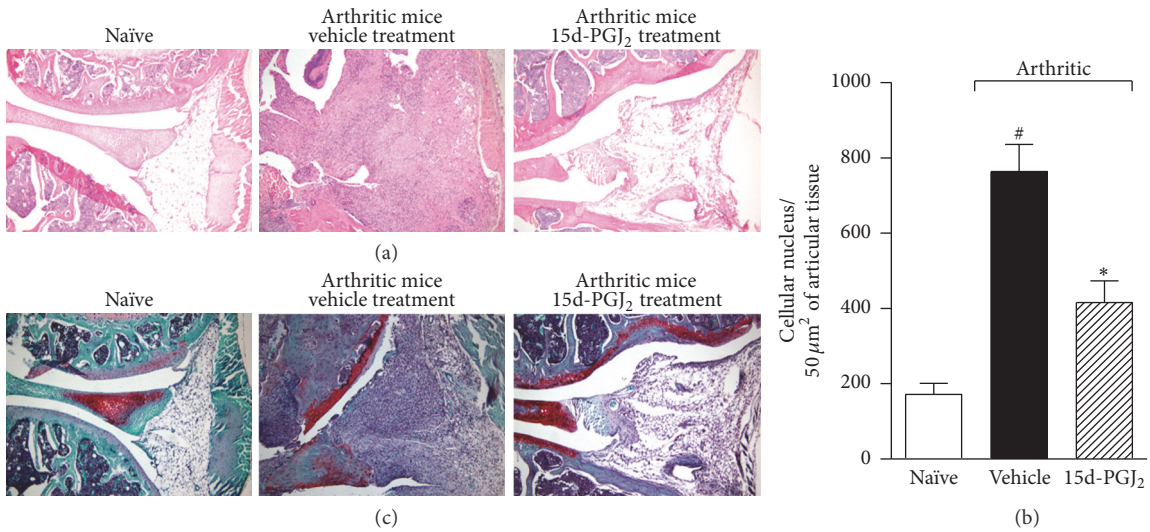

FIGURE 2: 15d-PGJ_2 treatment ameliorates articular inflammation. Naïve or collagen-immunized and challenged DBA/1 mice were injected s.c. daily with vehicle or 15d-PGJ_2 (1 mg/Kg) for 7 days. At the end of treatment, mice were euthanized, the articular joints were harvested, and histopathologic analysis was performed. Knee joint sections were stained with H&E (a) or with safranin-O (c), a proteoglycan red marker, to reveal profound cartilage damage in the vehicle-treated mice (less proteoglycan staining) and the preservation of cartilage in 15d-PGJ_2-treated mice. Quantification of cellular infiltrate was performed by ImageJ software (NIH, USA) in 40 fields with 400x magnification for each animal/group (b). Morphometric histologic examination revealed markedly less cellular infiltration in the 15d-PGJ_2 mice than in the PBS-treated group. $^{\#}P < 0.05$ compared with naïve group. $^{*}P < 0.05$ compared with arthritic mice treated with PBS (vehicle).

on CIA, we evaluated the expression of FOXP3, a transcription factor highly expressed in regulatory T cells that is related to the control of the immune response both *in vitro* and *in vivo* [18]. Interestingly, the induction of arthritis in mice did not increase FOXP3 mRNA expression in the draining lymph nodes of animals that received vehicle as treatment (Figure 5(a)). However, FOXP3 was highly expressed in the lymph nodes of arthritic animals treated with 15d-PGJ_2, and this increase was sixfold higher than the group of arthritic animals treated with vehicle.

To examine whether 15d-PGJ_2 affects FOXP3 expression in nTreg cells, we examined $CD4^+$ T cells after *in vitro* incubation with 15d-PGJ_2 (5 μM) and restimulation with collagen or plate-bound α-CD3. As shown in Figure 5(b), treatment with 15d-PGJ_2 did not interfere with $CD3^+CD4^+$ positivity compared to the vehicle. Similar effects were observed concerning the $CD4^+CD25^+$ population (Figure 5(b)). The expression of Treg markers like FOXP3 (Figure 5(c)), GITR (Figure 5(d)), and CTLA-4 (Figure 5(e)) in the $CD4^+CD25^+$ cells was also similar to those recovered from both the culture with the prostanoid and the culture with the vehicle. Unexpectedly, we detected the expression of markers characteristic of Tregs on the gate of $CD4^+CD25^-$, described as conventional T cells, and we observed a significant increase in the expression of FOXP3 (Figure 5(c)), GITR (Figure 5(d)), and CTLA-4 (Figure 5(e)), suggesting that 15d-PGJ_2 induces a regulatory T cell phenotype in conventional T lymphocytes.

3.5. Effect of 15d-PGJ_2 during T Cells Differentiation. Based on our finding that prostanoid treatment altered the phenotype of conventional T cells to a regulatory profile, additional experiments were conducted in purified $CD4^+CD25^-$ populations to obtain unequivocal evidence for the role of 15d-PGJ_2 in induced Treg (iTreg) generation. To generate iTregs, sorted $CD4^+CD25^-$ T cells from DBA/J naïve mice were cultured on plate-bound anti-CD3 mAb with anti-CD28 mAb, rmTGF-β, rmIL-2, anti-IFN-γ mAb, and anti-IL-4 mAb in the presence or absence of 15d-PGJ_2 (5 μM) or vehicle for 7 d. At the end of culture period, the cells were harvested and analyzed for FOXP3 expression by flow cytometry. As control groups, natural regulatory T cells ($CD4^+CD25^+$) or Th0 ($CD4^+CD25^-$) were cultured only in the presence of rmIL-2 for cell maintenance. As expected, FOXP3 was highly expressed in nTregs ($CD4^+CD25^+$). Under Treg-polarizing conditions, FOXP3 was also expressed in $CD4^+CD25^-$ cells cultured with vehicle but was enhanced when 15d-PGJ_2 was added to the culture (Figure 6(a)).

To further characterize the effect of prostanoid on Th17 differentiation, sorted naïve $CD4^+CD25^-$ T cells from DBA mice were cultured on plate-bound anti-CD3 mAb with anti-CD28 mAb, rmTGF-β, rmIL-1β, anti-IFN-γ mAb, anti-IL-4 mAb, and 15d-PGJ_2 (5 μM) or vehicle for 7 d. In the presence of 15d-PGJ_2, $CD4^+CD25^-$ Th17 differentiation was strongly reduced (Figure 6(b)). It is important to note that this effect on Th17 culture was not due to a cytotoxicity effect, as propidium iodide (PI+) positivity was not observed at any concentrations of 15d-PGJ_2 tested (Supplementary Figure 1). Altogether, the data suggest that 15d-PGJ_2 may modulate iTreg generation and inhibit the Th17 subset differentiation.

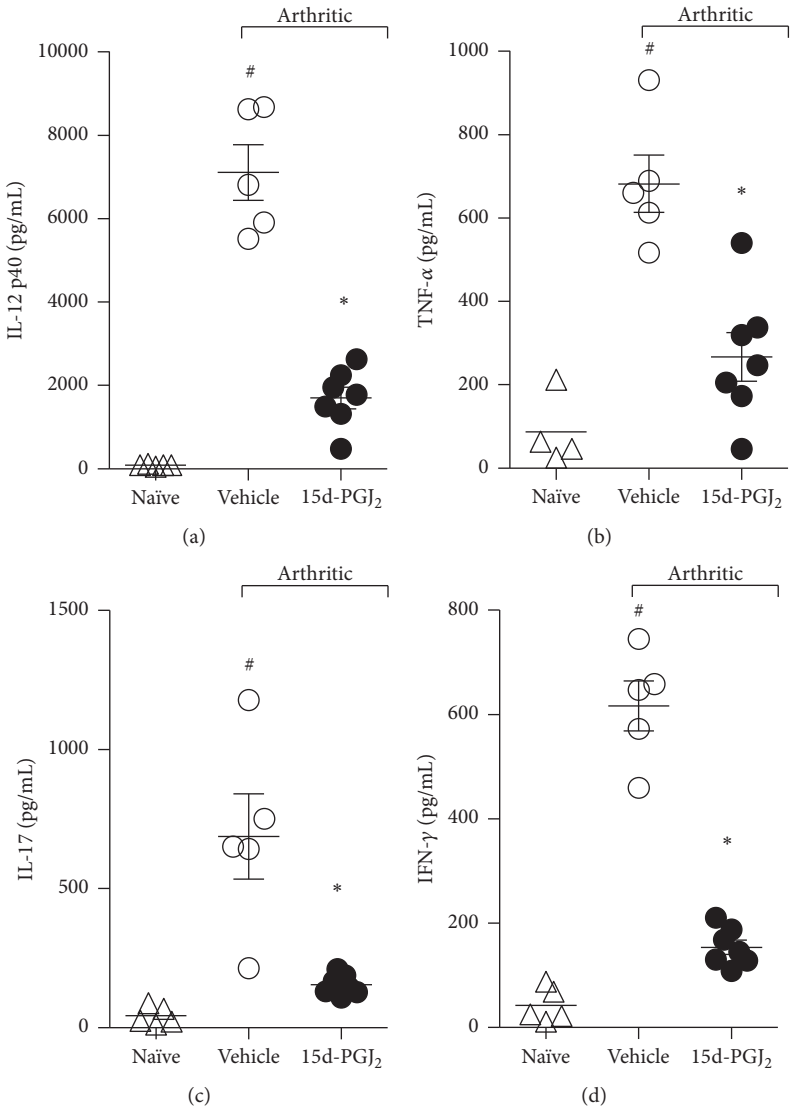

Figure 3: Decreased inflammatory cytokines in articular joints from 15d-PGJ_2-treated arthritic mice. Ankle joints from naïve (Δ) or PBS-(○) or 15d-PGJ_2-treated (●) arthritic mice were collected after 7 days of treatment for the determination of TNF-α (b), IFN-γ (d), IL-17 (c), and IL-12 (a) levels by ELISA in the homogenate supernatants. Results are expressed as the mean ± SEM, $N = 4$ (naïve) and 9-10 (arthritic groups). $^{\#}P < 0.05$ compared with naïve group. $^{*}P < 0.05$ compared with arthritic mice treated with PBS (vehicle).

4. Discussion

In the present study, we demonstrated that the expression of the PPAR-γ receptor is enhanced during experimental collagen-induced rheumatoid arthritis (CIA) and that its natural ligand, 15d-PGJ_2, reduces the severity of RA, characterized by a decrease in clinical scores, joint hyperalgesia, and edema as well as leukocyte migration to the joint tissue and cartilage degradation. The anti-RA effect of 15d-PGJ_2 was associated with its ability to induce iTreg and to inhibit Th17 subset polarization.

It is well accepted that the presence of various proinflammatory cytokines in the joint environment contributes to the pathophysiology of autoimmune arthritis. Among these cytokines, IL-12, TNF-α, and IFN-γ play a central role in the RA pathology [2, 19]. In the last two decades, IL-17, a cytokine released mainly by Th17 cells, has gained importance as a cytokine that orchestrates arthritis pathology. For instance, the presence of IL-17 has been demonstrated in the synovial fluids and tissues of RA patients as well as in several experimental RA models. IL-17 mediates most RA events, including leukocyte recruitment to the joint as well as joint pain [20]. Moreover, IL-17 induces the release of several well-known proinflammatory cytokines, including TNF-α and chemokines [19]. The therapeutic treatment of the arthritic mice with 15d-PGJ_2, administered daily subcutaneously for one week after the onset of disease, blocked the production of all abovementioned cytokines in the joint exudate. Furthermore, the presence of the ROR-γt transcription factor, which is related to Th17 differentiation, was also inhibited in the draining lymph nodes by this treatment. Moreover, 15d-PGJ_2

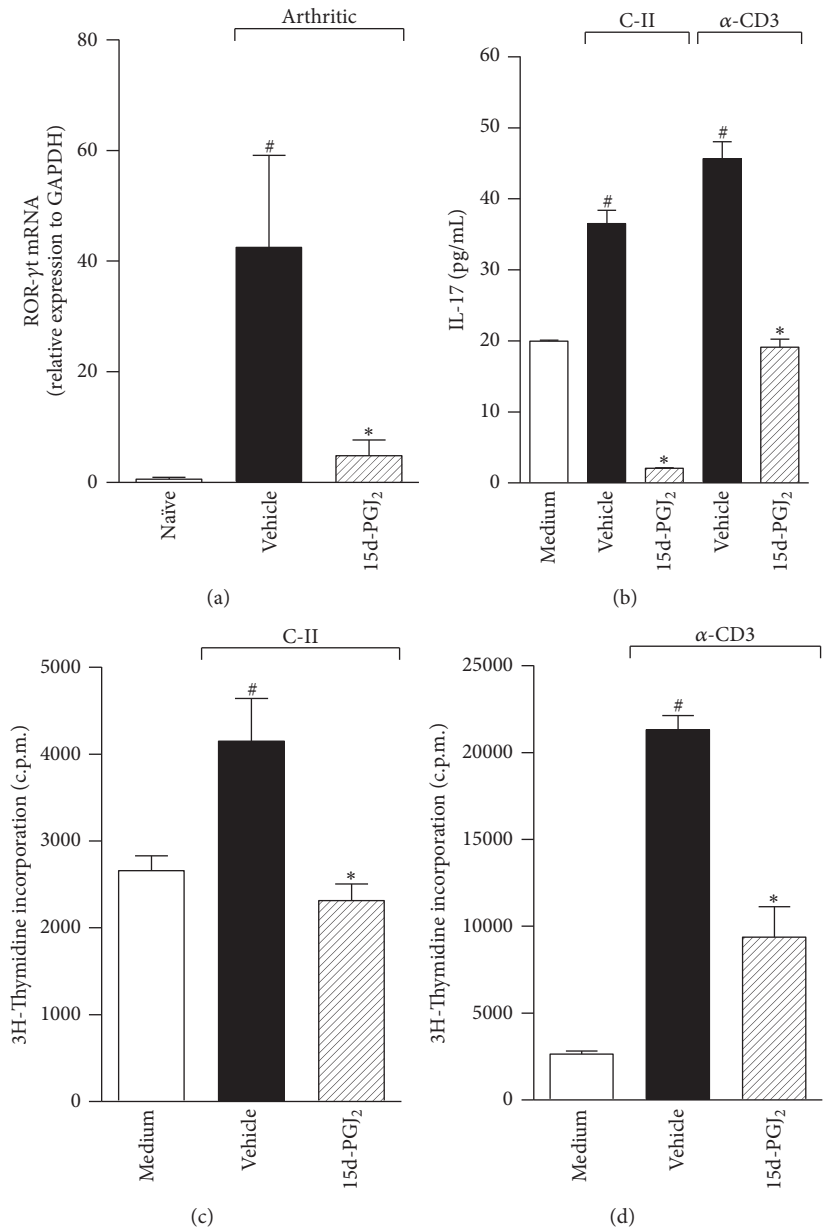

FIGURE 4: 15d-PGJ_2 suppresses the collagen-induced Th17 immune response. ROR-γt mRNA expression was quantified by real-time PCR in draining lymph nodes from naïve mice (white bar) or collagen-immunized and challenged DBA/1 mice treated with vehicle (PBS) (black bar) or – 15d-PGJ_2 (1 mg/Kg) (hatched bar) for 7 days (a). $^{\#}P < 0.05$ when compared with naïve mice. $^{*}P < 0.05$ when compared with vehicle (PBS). 15d-PGJ_2-pretreated nonadherent cells (2×10^6 cells/mL) (1 hour before) from draining LNs from the mice above were stimulated *in vitro* with C-II (5 μg/mL) or plate-bound α-CD3 (5 μg/mL) for 96 h. Culture supernatants were harvested to measure IL-17 (b) levels from C-II- or α-CD3-stimulated cultures. The specific C-II (c) or αCD3 polyclonal (d) stimuli proliferation assay was assessed by overnight [^{3}H]thymidine incorporation. The results are expressed as the mean ± SEM obtained from triplicate samples from two or three independent experiments (N = 3 per group). $^{\#}P < 0.05$ when compared with medium. $^{*}P < 0.05$ compared with vehicle (PBS).

selectively suppressed effector cells, including Th17, as demonstrated *in vitro* by polyclonal (α-CD3) or specific (collagen-II) stimuli, blocking both IL-17 production and lymphocyte proliferation. Similarly, Klotz and coworkers have reported that pioglitazone, a synthetic PPAR-γ agonist, inhibits the differentiation of Th17 cells and thereby suppresses experimental autoimmune encephalitis [9]. In a murine model of allergic airway inflammation, the PPAR-γ agonists pioglitazone and rosiglitazone reversed the pathophysiological features of asthma by suppressing the release of IL-17 into the lung [12]. Regarding arthritis, at least to our knowledge, this is the first study to demonstrate the therapeutic effects of 15d-PGJ_2 in a mouse model of rheumatoid arthritis. In rats with adjuvant-induced arthritis (AIA), pioglitazone decreased bone destruction by controlling the circulating and local expression of IL-17, with a subsequent decrease in the RANKL/OPG

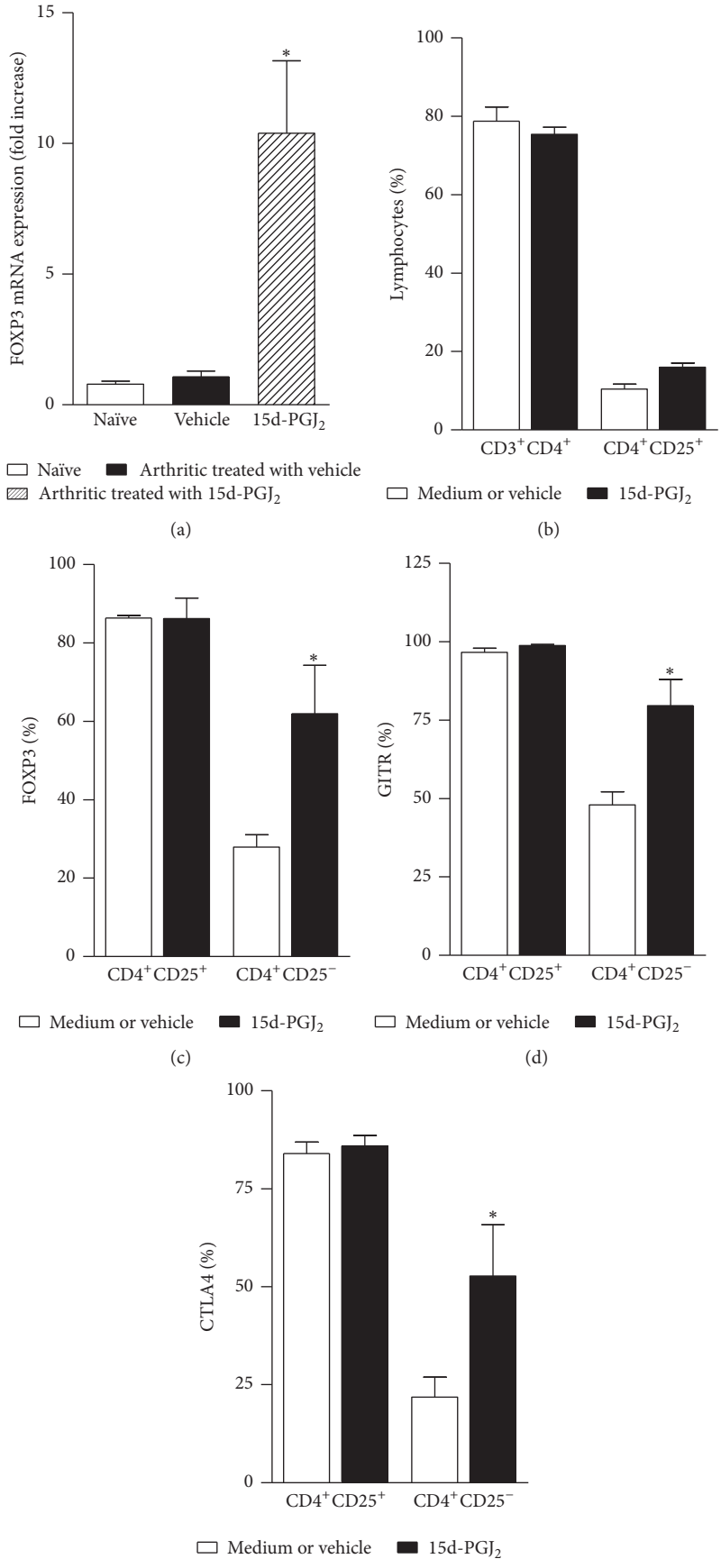

Figure 5: Continued.

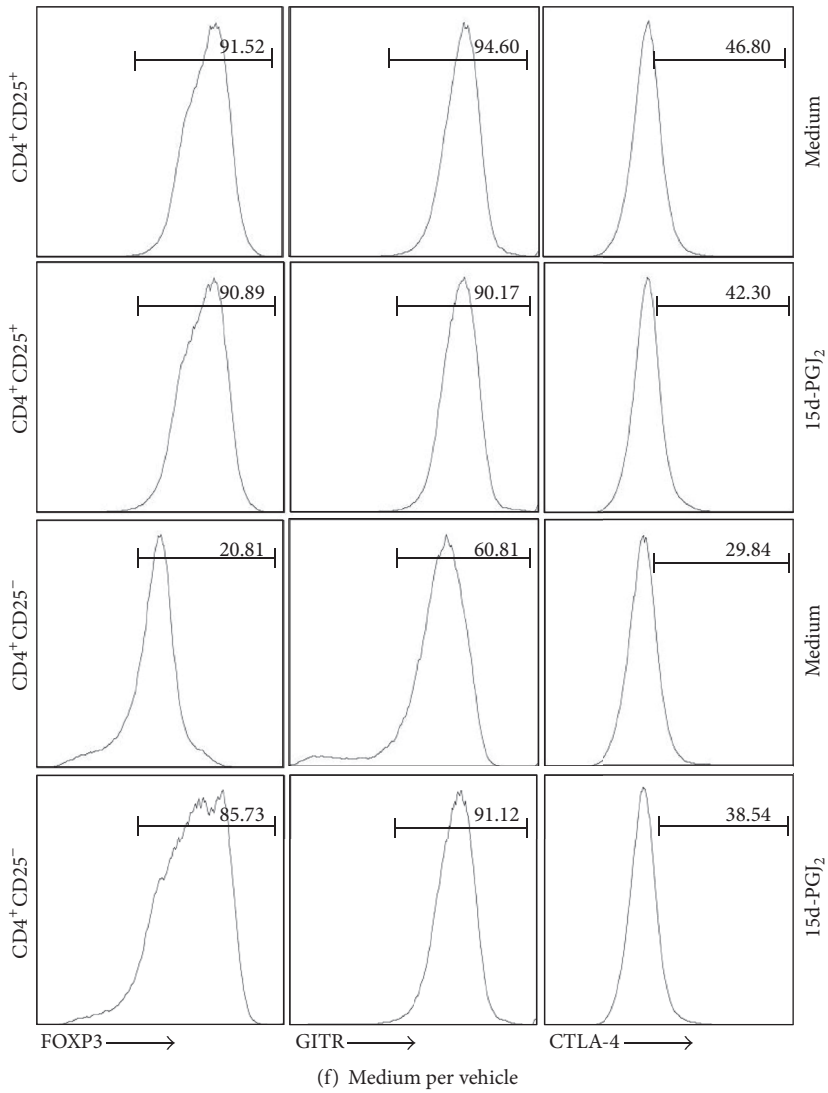

Figure 5: 15d-PGJ_2 induces regulatory T cell markers in conventional T cells. (a) FOXP3 mRNA expression was quantified by real-time PCR in draining lymph nodes from naïve mice (white bar) or collagen-immunized and challenged DBA/1 mice treated with vehicle (black bar) or 15d-PGJ_2 (hatched bar) after 7 days of treatment. Results are presented as the mean ± SEM, $N = 6$; $^{*}P < 0.05$ compared with PBS-treated group. Total cells (2×10^6 cells/mL) from the draining lymph nodes from naïve or arthritic animals were *in vitro* incubated with 15d-PGJ_2 (5 μM) (black bars) or vehicle (DMSO) (white bars) for 96 hours on plates coated with α-CD3. The nonadherent cells were phenotyped by flow cytometry using specific antibodies: anti-CD3 conjugated with FITC, anti-CD4 conjugated with PerCP, and anti-CD25 conjugated with APC-Cy7 (b) and anti-FOXP3 (c), anti-GITR (d), and anti-CTLA-4 (e) conjugated with PE. Lymphocytes were gated on $CD4^+CD25^+$ or $CD4^+CD25^-$, and the population expressing the markers described above was subsequently analyzed. In (f), representative histograms of FOXP3, CTLA-4, and GITR are shown in each box. The values above are expressed as the mean ± SEM, which are representative of quadruplicate samples from two independent experiments ($N = 4$). $^{\#}P < 0.05$ compared with $CD4^+CD25^-$ group control (vehicle).

ratio [21]. We hypothesized that the potential mechanism by which 15d-PGJ_2 decreased T cell proliferation could be mediated by inhibition of IL-2 secretion. Mechanistic studies indicate that PPARs intrinsically influence T helper differentiation and function and impair T cell proliferation through an IL-2 dependent mechanism involving repression of NFAT activity [7, 22]. Thus, the inhibition of the production of proinflammatory cytokines, especially IL-17, by 15d-PGJ_2 treatment is likely a crucial step in limiting the tissue damage observed in RA. Moreover, it is reasonable to suggest that the inhibition of PPAR-γ may represent a new therapeutic strategy for RA.

The increased expression of PPAR-γ in arthritic was concomitant with ROR-γt expression since such receptor is enhanced in most activated leukocytes, including Th17 subset, displaying a repressor role on inflammatory condition in promoting tissue repair and recovering the homeostasis [6]. Upon ligand binding, PPAR-γ heterodimerizes with the retinoid X receptor and binds to the PPAR response elements (PPRE) located in the promotor region of target genes [23,

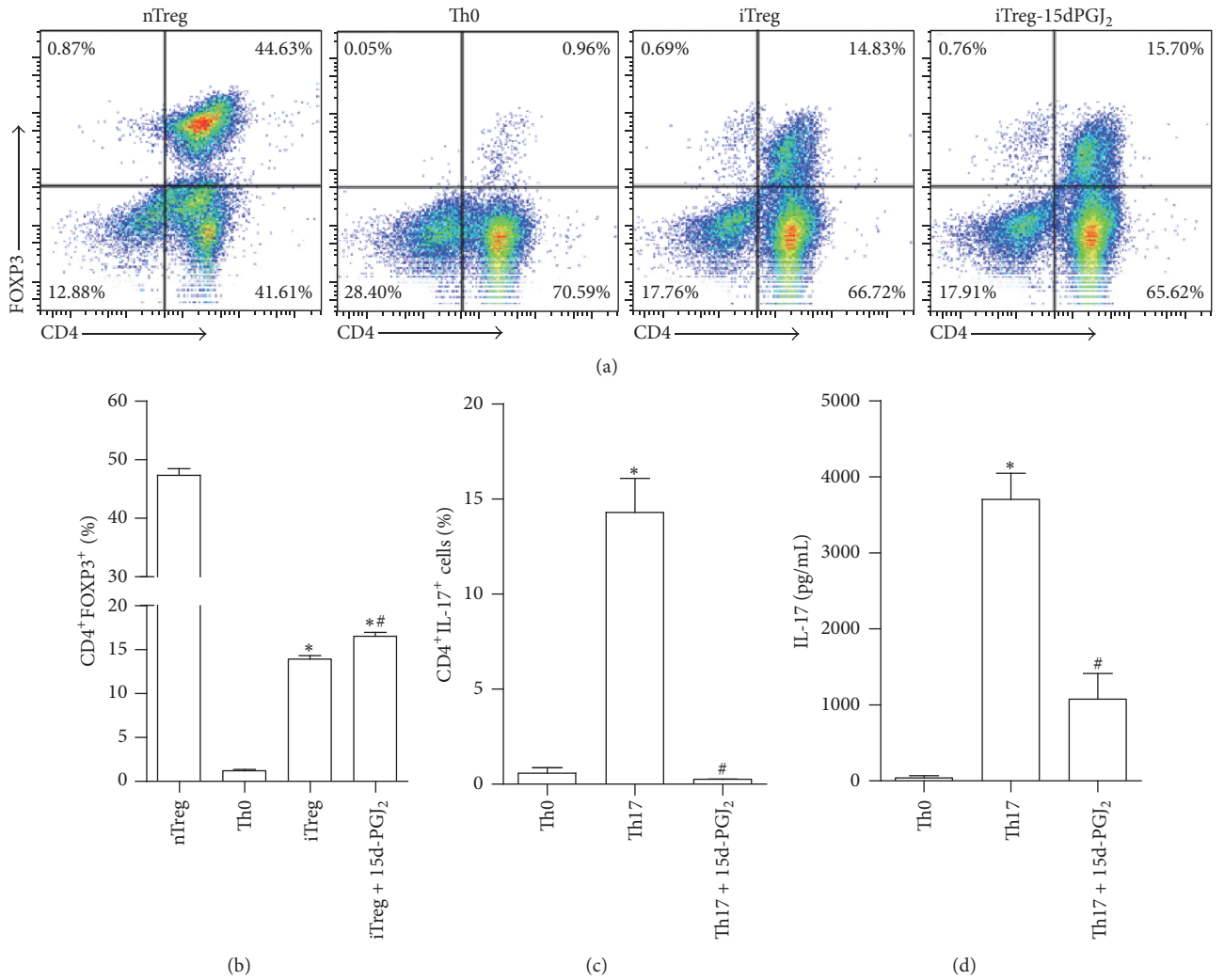

FIGURE 6: 15d-PGJ_2 altered the profile of $CD4^+CD25^-$ cells under polarizing conditions. Isolated $CD4^+CD25^-$ cells from naïve mice were cultured under Treg ((a)/(b)) or Th17 ((c)/(d)) polarizing conditions with or without 15d-PGJ_2 (5 μM). Natural Treg (nTreg, $CD4^+CD25^+$) or Th0 ($CD4^+CD25^-$) cells were used as positive (for Treg) and negative differentiation controls. The bars represent the percentage of $TCD4^+$ cells expressing FOXP3 or IL-17. In (d), IL-17 levels were measured into supernatant culture from Th17 polarizing condition by ELISA assay. The results are expressed as the mean ± SEM obtained from triplicate samples from one of three independent experiments ($N = 3$ per group). $^*P < 0.05$ relative to the vehicle group. $^*P < 0.05$ compared with Th0; $^\#P < 0.05$ compared with iTreg (b). $^*P < 0.05$ compared with Th0; $^\#P < 0.05$ compared with Th17 (c-d).

24]. Additionally, the anti-inflammatory effects of PPAR-γ are mediated by negative pathway of proinflammatory cell signaling, for example, stabilization of corepressor complexes, such as nuclear corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) [23, 25]. Recently, it was described that ciglitazone inhibited both the proliferation of IL-17-producing cells and the expression of CCNB1, which regulates the cell cycle presumably by inhibiting cyclin B expression [26].

Although 15d-PGJ_2 has high affinity for PPAR-γ and such receptor is highly expressed during activated phase of CIA, we not discard the possibility of 15d-PGJ_2 to modulate the inflammation in PPAR-γ-independent mechanisms. Reports have shown that 15d-PGJ_2 can repress some genes expression through the direct binding of ERK-MAPK and NFκB [27, 28]. Furthermore, the proinflammatory production of NOS-2, metalloproteinase-2 (MMP-2), and MMP-9 as well as IL-6 and TNF-α in cultured cardiomyocytes infected with *Trypanosoma cruzi*, a protozoa parasite, was inhibited by administration of 15d-PGJ_2, but rosiglitazone, a synthetic PPAR-γ agonist, was inefficient in inhibiting such mediators [29]. Up to date, there is no evidence of whether 15d-PGJ_2 may selectively suppress Th17 cell differentiation in PPAR-γ-independent manner and the molecular mechanism remains to be investigated.

The anti-inflammatory activity of 15d-PGJ_2 may be related to regulatory T cell generation. The levels of the classical transcription factor of regulatory T cells, FOXP3, were increased in the lymph nodes of treated animals. Several studies have reported that PPAR-γ agonists enhance the induction

and function of Tregs in mice. Wohlfert and colleagues demonstrated that ciglitazone promoted the conversion of naïve T cells into $CD4^+FOXP3^+$ cells *in vitro* [30]. PPAR-γ-deficient Tregs exhibit an impaired ability to prevent effector T cells-induced colitis [31]. Furthermore, Iwami et al. showed that PPAR-γ agonists induce Tregs and prolong the survival of cardiac allografts [32]. Interestingly, we observed that the addition of 15d-PGJ_2 into cell culture did not increase the numbers of nTregs ($CD4^+CD25^+FOXP3^+$) but increased the levels of FOXP3, CTLA-4, and GITR in $CD4^+CD25^-$ cells, suggesting that the activation of PPAR-γ primarily induces the generation of iTregs. Furthermore, prostanoid treatment promoted a potent suppressive function *in vitro*. These findings are consistent with those obtained by Lei and colleagues, who demonstrated that PPAR-γ agonists, including 15d-PGJ_2, induce and maintain FOXP3 expression in the $CD4^+CD25^-$ subpopulation of human lymphocytes [33]. Furthermore, iTreg can suppress the proliferation of effector T cells in a cell contact-independent fashion or through the production of anti-inflammatory cytokines such IL-10 [34, 35] and TGF-β [36]. Thus, expanding Treg cells during the ensuing chronic phase of disease may prevent collateral damage by suppressing the Th subset, especially Th17 cells. For unequivocal evidence of the effect of 15d-PGJ_2 upon Treg generation and the suppression of Th17 cells, we submitted sorted naïve $CD4^+CD25^-$ cells to polarizing conditions for both subsets. The prostanoid treatment promoted Treg generation by increasing FOXP3 expression on $CD4^+CD25^-$ cells but restricted Th17 differentiation. Thus, the preservation of the joints of animals treated with prostanoid may be due to Treg cells.

Presumably, an aberrant Th17 response in the inflamed tissue may reflect Treg function during RA. Several studies have reported that Treg $CD4^+CD25^{high}$ cells are present in the inflamed synovium of arthritic patients and that their suppressive function is normal *in vitro* [37–39]. However, some studies have demonstrated that these cells exhibit a defect in their ability to suppress the proliferation of effector T cells present in the inflamed joint [37, 40]. Moreover, adoptive transfer of Treg cells may fail to heal well-established autoimmune diseases, indicating that, under certain chronic inflammatory conditions, Tregs are unable to mediate the immunosuppressive effect. Indeed, Tregs cultured with proinflammatory cytokines lose their capacity to inhibit responder cell proliferation and cytokine production [41]. Recently, our group demonstrated that the refractoriness of RA patients to methotrexate, the first-line pharmacotherapy for RA, is closely associated with a commitment to Treg expansion and function [42]. Thus, it is plausible to suggest that pharmacologic strategies that reinforce the suppressive function and/or the induction of Treg cells could represent a therapeutic target for the treatment of RA. Herein, we presented evidence that the blockage of Th17 with concomitant Treg generation by 15d-PGJ_2 may be an interesting alternative to ameliorate the clinical symptoms of RA.

5. Conclusion

In the present study, we demonstrated that 15d-PGJ_2 presents a potential therapeutic effect on collagen-induced rheumatoid arthritis (CIA). Such prostanoid suppresses the inflammatory process by promoting a regulator profile on T cells and in dampening the differentiation of Th17 cells. In last instance, it protect the articular joint from inflammatory insult.

Abbreviations

iTreg: Induced Treg
LN: Lymph node
nTreg: Natural Treg
Teff: Effector T cell
Treg: Regulatory T cell
WT: Wild type.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Review Article

The Immune System in Tissue Environments Regaining Homeostasis after Injury: Is "Inflammation" Always Inflammation?

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Inflammation is a response to infections or tissue injuries. Inflammation was once defined by clinical signs, later by the presence of leukocytes, and nowadays by expression of "proinflammatory" cytokines and chemokines. But leukocytes and cytokines often have rather anti-inflammatory, proregenerative, and homeostatic effects. Is there a need to redefine "inflammation"? In this review, we discuss the functions of "inflammatory" mediators/regulators of the innate immune system that determine tissue environments to fulfill the need of the tissue while regaining homeostasis after injury.

1. Introduction

Inflammation is one of the major danger control programs of tissue pathology conserved during evolution till date with a major aim to resolve the infection, repair the tissue damage, and regain the state of tissue homeostasis [1, 2]. It is a highly complex but still a very well-coordinated process, classically triggered by infection or tissue injury. Historically, "inflammation" was initially defined based on the clinical representations by Hippocrates as *calor*, *rubor*, tumor, and *dolor* [3]. This definition was challenged by the discovery of microscope in the 19th century, and the microscopic presence of leukocytes at the site of infection or injury was called "inflammation" since then [4]. However, this simplistic definition of "inflammation" does no longer hold true in the 21st century mainly because of the advancements in immunology and leukocyte biology in the last decade. We now know that leukocytes present numerous immunoregulatory phenotypes, for example, M2 macrophages, regulatory T and B cells, and fibrocytes, having anti-inflammatory functions. This implies that the presence of leukocytes observed by pathologists at sites of infection or injury does not necessarily indicate "inflammation," at least without further characterizing their functional phenotypes. As such, we now define "inflammation" based on the presence of proinflammatory leukocyte phenotypes along with the expression of proinflammatory cytokines.

A successful inflammatory response eliminates the trigger followed by a resolution of inflammation and tissue repair by numerous anti-inflammatory cytokines as well as lipid mediators [5–8]. However, a persistent injurious trigger shifts the homeostatic set points fetching several changes in the initial inflammatory process (chronic inflammation), for example, replacement of neutrophils with macrophages and T cells and subsequent formation of granulomata or tertiary lymphoid tissues. In case these cellular effectors fail to control the injurious trigger, collateral tissue damage occurs [9–11]. Moreover, chronic inflammation can also arise as a result of autoimmune responses [9, 11]. Regardless of the cause, inflammation supposedly evolved to restore homeostasis. In this review, we discuss how different mediators of inflammation, in particular, of the innate immune system, set tissue environments to resolve inflammation and reinforce tissue repair, by promoting either regeneration or fibrosis in order to regain homeostasis after injury (Figure 1).

2. Resolution of Inflammation

An acute inflammatory response is followed by the resolution phase. The processes to return to tissue homeostasis, that is, catabasis [12], are governed by innate immune cells and specific mediators produced by them. These processes involve neutrophil apoptosis and their phagocytic removal via efferocytosis, clearance of proinflammatory dead cells and cytokines, and recruitment or phenotype switching of macrophages to anti-inflammatory phenotype [13]. Neutrophil-derived microparticles can also trigger the resolution of inflammation [13, 14]. Factors that mediate resolution include interleukin- (IL-) 10 and TGF-β, as well as lipid mediators, for example, lipoxins, resolvins, protectins, and maresins, collectively termed as specialized proresolving mediators (SPMs) [6, 15]. Within minutes after tissue injury prostaglandin and leukotriene synthesis from arachidonic acid metabolism occurs at the site of inflammation leading to the recruitment of neutrophils as a result of the chemotactic gradient, increased blood flow, and vascular permeability [16]. This is often followed by the class switching of lipid mediators, in which arachidonic acid metabolism switches from the production of leukotrienes to anti-inflammatory lipoxins, thus sending the "stop" signal to neutrophils recruitment and begins the end of the acute inflammatory response [17]. Lipoxins and resolvins stimulate the nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages [18]. SPMs counterregulate the proinflammatory mediators and thus reduce the magnitude and duration of inflammation and tissue regeneration [12, 19].

Apart from limiting neutrophil recruitment, SPMs also help to increase natural killer (NK) cells mediated neutrophil apoptosis and subsequent efferocytosis by macrophages [20]. They potently inhibit the release of proinflammatory cytokines from the group 2 innate lymphoid cells (ILCs) [20] and increase IL-10 production by macrophages as well as induce M1 to M2 macrophage phenotype switch [21]. In addition to SPMs, the complement system also contributes to the resolution of inflammation by enhancing efferocytosis of apoptotic cells [22, 23]. The continuous phagocytosis of apoptotic cells, regulated by the mitochondrial membrane protein Ucp2 [24], stimulates monocytes to release IL-10 and TGF-β further promotes the switch toward an anti-inflammatory M2 macrophage phenotype [25, 26]. Recently, resolvin D1 has been demonstrated to trigger GPR32 to polarize macrophages toward the proresolving M2 phenotype [27]. Furthermore, IL-10 is an important cytokine with anti-inflammatory functions [28]. For example, in mouse models of acute kidney injury, IL-10 administration has a beneficial effect by inhibition of leukocyte infiltration and inflammatory renal cell death [29]. It also influences T cells by attenuating proliferation of CD4+ T cells and their cytokine production [30]. The tissue-resident dendritic cells (DCs) also promote the resolution of inflammation by producing pentraxin-3 (PTX3) which inhibits P-selectin on the vascular endothelial cells and thus inhibits immune cell recruitment to sites of injury [31–33]. Moreover, neutrophils released the prestored PTX3 in the early phase of acute myocardial infarction that bind to activated circulating platelets and dampen their proinflammatory response [34], whereas PTX3 also aggregated with histones and protected from histone-mediated endothelial cytotoxicity in sepsis [35, 36]. Furthermore, PTX3 suppressed complement dependent inflammation as well as reduced tumor infiltration by macrophages [37].

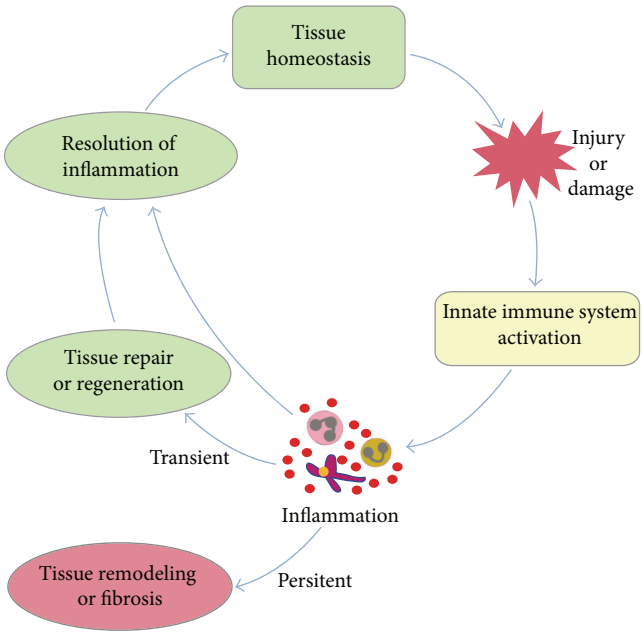

Figure 1: The role of the innate immune system in regaining tissue homeostasis. An injury disturbs the tissue homeostasis and activates the innate immune system leading to the recruitment of several immune cells at the site of injury. These immune cells secrete cytokines, growth factors, and enzymes to establish an inflammatory milieu. They also secrete anti-inflammatory and proregenerative cytokines to promote resolution of inflammation as well as tissue repair. A transient inflammation is often helpful to get rid of the cause of the tissue injury and return to homeostasis. However, an uncontrolled or persistent inflammation promotes tissue remodeling and fibrosis.

Group 3 ILCs gets activated and produces IL-22 after an intestinal epithelial injury suggesting that inflammation can override injury by promoting tissue regeneration [38]. Moreover, IL-22-producing ILCs prevented systemic inflammation during chronic diseases by promoting anatomical containment of lymphoid-resident commensal bacteria [39]. Similarly, the redox modification of high mobility group box 1 (HMGB1), a danger associated molecular pattern (DAMP) released after tissue injury as well as by macrophages and monocytes, regulated its proinflammatory functions during the resolution of inflammation and prevented excessive acetaminophen-induced hepatic injury [40, 41]. Together, immune cells, as well as mediators released by them, promote resolution of inflammation in order to reestablish the homeostasis after injury.

3. Tissue Regeneration and Repair

The immune system is instrumental in supplying growth factors and cytokine signals that orchestrate tissue repair. For

example, the tissue-resident macrophages originated from yolk sac-derived erythromyeloid progenitors that possess the capacity to self-replenish [42, 43], while bone-marrow-derived circulating monocytes differentiate into tissue macrophages [44], but both are activated during injury. Blood-derived young monocytes/macrophages have enhanced remyelinating activity compared to old macrophages in the central nervous system [45]. Although M2 macrophages are the main driver of the resolution of inflammation, tissue repair, and scar formation, the M1 macrophages clear cellular debris in order to prevent the persistence of toxic and immunogenic material at the site of injury. Therefore, depletion of M1 macrophages resulted in impaired healing and regeneration after myocardial as well as skeletal muscle injuries [46, 47]. In addition, M1 macrophages also activated proliferative myogenesis via IL-6, TNF-α, and IL-1β whereas M2 macrophages supported myogenic differentiation via TGF-β production during skeletal muscle regeneration [47, 48].

Moreover, infiltrating eosinophils secreted IL-4 to induce proliferation of fibro/adipocyte progenitor cells, which promoted clearance of necrotic debris and skeletal muscle regeneration [49]. The CXCL12-CXCR4 pathway regulates the recruitment of progenitors, the unipotent proliferative cells with a capacity of self-renewal [50], at the site of injury [51]. Other mediators of the innate immune system that induced progenitor cells proliferation and regeneration include leukotriene C4, which activated radial glial cell proliferation and neurogenesis either upon or without an injury [52], oncomodulin derived from neutrophils, and macrophages which promoted the optic nerve regeneration [53, 54]. Furthermore, macrophages derived Wnt suppressed Notch signaling and thus regulated the fate of hepatic as well as renal progenitor cells after liver and kidney injury, respectively [55, 56]. Macrophage-derived Wnt7b also stimulated epithelial responses and, thus, regarded critical for kidney repair and regeneration [57]. In the acidic tissue environments after skin, liver, and lung injury and arterial thrombosis, neutrophils and macrophages derived PTX3 promoted remodeling of the fibrin-rich inflammatory matrix ensuring normal tissue repair [58]. In addition, the complement system is also instrumental in promoting tissue repair and regeneration by inducing growth factors as well as disposal of dead cells [23]. In particular, C3a and C5a activated NF-κB/STAT-3 and enhanced hepatocyte regeneration after liver injury [59]. Recent reports showed that the delayed postinjury administration of C5a inhibited caspase-3 mediated neuron apoptosis leading to improved regeneration and functional recovery after murine spinal cord injury [60], as well as administration of C3a retina regeneration via STAT-3 activation in the progenitor cells present in the eye [61].

Apoptotic cells released after tissue injury promoted angiogenic properties of macrophages by releasing prostaglandin E2, which induced endothelial-derived progenitors to angiogenesis and vascular repair during skeletal muscle regeneration [62–64]. In addition, proliferation and differentiation of renal progenitor cells were also enhanced by the Toll-like receptor- (TLR-) 2-agonistic DAMPs released after tissue injury [65–67]. Several recent data suggests additional mechanisms of DAMP-driven tissue regeneration. For example, TLR4-agonistic DAMPs activated the interstitial mononuclear phagocytes to secrete a proregenerative cytokine IL-22 [68, 69] to promote tubular cell regeneration after injury by activating the JAK/STAT3 and ERK1/2 signaling pathway [68, 70]. Group 3 ILCs also produced IL-22 after an intestinal injury to promote the intestinal stem cell-mediated epithelial regeneration [71]. IL-22 mediated protection and regeneration were also observed in experimental models of hepatic, pancreatic, and thymic injuries [72–75]. In addition, a mast cell-specific tryptase, mouse mast cell protease (mMCP) 6, directly cleaves fibronectin and collagen IV and, therefore, suppressed scars and promoted functional recovery after spinal cord injury [76]. Platelets contributed to liver regeneration by secreting serotonin in mice as well as humans [77, 78]. Group 2 ILCs also promoted lung-tissue homeostasis after infection with influenza virus by producing a growth factor Amphiregulin [79]. After an injury to skeletal muscles, IL-33 recruited a special population of regulatory T cells (Tregs) to the injured muscles that produced Amphiregulin and improved the muscle repair [80–82]. Together, this illustrates that the innate immune system and its mediators do not only contribute to the immune injury but also to the immune-mediated repair or regeneration after injury as a part of a danger control response [10, 83] (Figure 2).

4. Tissue Remodeling and Fibrosis

The well-defined chronology of inflammatory events is essential for optimal repair. However, an overactivated immune response leads to tissue remodeling rather than tissue regeneration, which is clinically termed as tissue fibrosis. Fibrosis is characterized by excess deposition of extracellular matrix (ECM) due to the accumulation and activation of fibroblasts and myofibroblasts. Inflammatory cells of the immune system, as well as factors released by them, facilitate fibrosis. For example, tissue injury is always followed by altered vascular permeability to enhance the neutrophils recruitment to the site of injury. The delayed clearance of neutrophils from the site of injury further exacerbates the injury [84]. Neutrophils count is in fact used as a prognostic marker for cardiac remodeling [85]. Neutrophils are known to increase oxidative stress as well as release a number of enzymes like matrix metalloproteinases (MMPs), elastase, and cathepsins which contribute significantly to the process of fibrosis [86–88]. Apart from neutrophils, platelets can also respond to the state of infection or inflammation through activation of TLRs [89, 90]. The factors derived from platelets, for example, platelet-derived growth factor (PDGF), is a potent chemotactic agent, whereas TGF-β drives fibroblast proliferation and activation [91]. Moreover, the factors involved in coagulation can also contribute significantly to fibrosis, for example, factors VII, IX, and X [92–94]. The coagulation system and complement system are linked very closely, often involving a cross talk, to maintain the tissue homeostasis [23, 95]. For example, in the absence of C3, thrombin replaces the C3 dependent C5 convertase and directly cleaves C5 to generate the biologically active C5a [96], which induced fibrosis in lungs, liver,

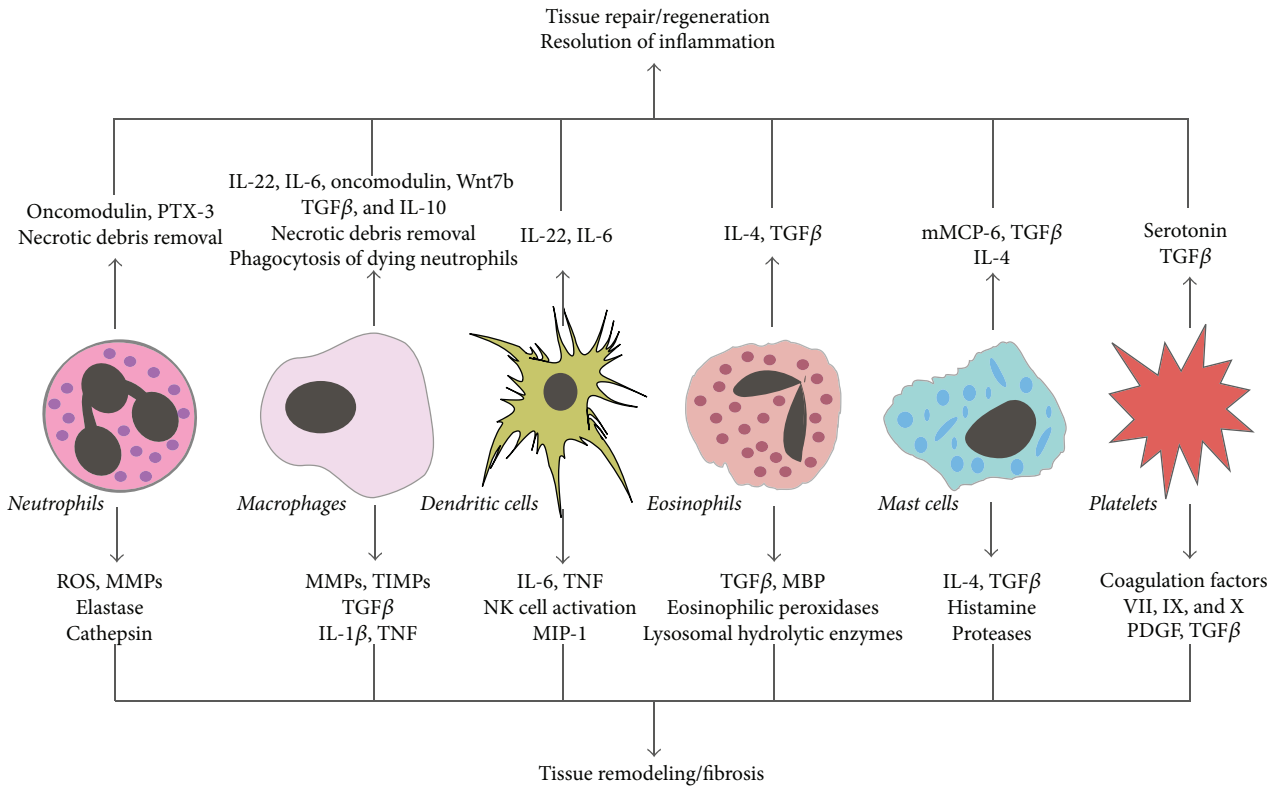

Figure 2: Mediators of innate immune system in regaining tissue homeostasis. Innate immune cells secrete several cytokines, growth factors, and enzymes, which promotes either resolution of inflammation and tissue repair/regeneration or tissue remodeling/fibrosis. PTX3: pentraxin 3, ROS: reactive oxygen species, IL: interleukin, TGF: transforming growth factor, MMP: matrix metalloproteinase, TIMP: tissue inhibitor of matrix metalloproteinase, TNF: tumor necrosis factor, MIP: macrophage inhibitory protein, MBP: major basic protein, mMCP: mouse mast cell protease, and PDGF: platelet-derived growth factor.

pancreas, and kidney after injury [97–100]. In addition to C5a, C3a has also been implicated in renal fibrosis [101].

Other cells of the innate immune system, for example, eosinophils and mast cells, also contribute to fibrosis. Eosinophils produce TGF-β, major basic protein (MBP)-1, eosinophilic peroxidase as well as granule proteins, and lysosomal hydrolytic enzymes which are implicated to be a part of fibrosis process [102]. Mast cells produce various proteases, vasoactive factors like histamine, cytokines, and TGF-β during the tissue injury and fibrosis [103]. IL-4, one of the major products of mast cell activation, contributes to the cardiac fibrosis [104]. Moreover, mast cell deficient mice are protected against pulmonary as well as cardiac fibrosis [105, 106]. Among the various immune cells, the macrophages are essential for efficient wound healing [107–111]. Macrophages are the main source of MMPs and tissue inhibitor of metalloproteinases (TIMPs) [109, 112, 113]. The balance between MMPs and TIMPs is crucial for maintaining the composition of ECM. Apart from MMPs and TIMPs, macrophages also contribute to the production of TGF-β, the most significant factor involved in fibrosis [114]. TGF-β regulates fibroblast activation, differentiation, and proliferation [115]. It also upregulates ECM genes and suppresses genes associated with MMPs, thus causing increased deposition of matrix. TGF-β promotes collagen synthesis and the expression of profibrotic genes such as type I collagen and connective tissue growth factor (CTGF) [15, 116]. Furthermore, TGF-β is also an anti-inflammatory factor; therefore, its early inhibition is associated with increased mortality, increased chemokine expression, and leukocyte infiltration while its inhibition during the resolution phase resulted in improved survival and reduced tissue fibrosis [117–122]. Activated macrophages induce production of various cytokines and factors like interleukins (e.g., IL-1β) and TNF-α, which drive further inflammation and fibrosis by enhancing ECM production as well as upregulating expression of TGF-β [123, 124]. Depletion of macrophages during the early phase of tissue injury ameliorated fibrosis, while delayed depletion of macrophages during the resolution phase exaggerated fibrosis with persistence of profibrotic cellular and matrix components [110, 111, 125]. Although recent studies demonstrated an association between macrophages derived PTX3 and tissue fibrosis in nonalcoholic fatty liver disease as well as in lung fibrosis, whether PTX3 causes fibrosis or not is still unclear [126, 127].

Along with macrophages, DCs are the primary determinants of the cytokine and chemokine milieu during fibrogenesis [128, 129]. For example, IL-6 and TNF-α produced by DCs have pleiotropic effects on liver fibrosis [128]. Furthermore, they also activated NK cells to produce TNF-α and, therefore, elevate the inflammatory environment in fibrotic

livers [128]. Group 3 ILCs promoted bleomycin-induced pulmonary fibrosis by secreting IL-17 [130, 131], whereas IL-25 induced expansion of the group 2 ILCs within the lungs, which promoted pulmonary fibrosis via IL-13 dependent mechanism [132]. Together, the innate immune system and its mediators contribute to tissue remodeling and fibrosis (Figure 2).

5. Conclusions and Future Perspectives

Maintaining tissue morphology is essential to maintain tissue function, that is, homeostasis. An injury or damage affects the structural integrity of the tissue implying a loss of tissue function, and, therefore, the structural and functional recovery, that is, regaining homeostasis after injury, is the ultimate goal. The inflammatory mediators of the innate immune system are important regulators of tissue homeostasis. They modulate tissue environments at all phases of the homeostatic imbalance, for example, promotion as well as the resolution of inflammation, tissue regeneration, and tissue remodeling/fibrosis.

Our understanding of inflammation biology has increased over the last few decades and has gone far beyond the basic concept of inflammation that was originally introduced by Hippocrates. The advancements in microscopic as well as flow associated cell sorting (FACS) techniques have allowed us to understand and redefine the "inflammation." This progress has also raised several questions, for example, what are the molecules or signals that regulate the function of the innate immune cells, what are the critical mechanisms that regulate the balance of different populations of these cells in the specific phase after injury, and how to modulate the behavior as well as balance of these cells in each phase after injury to enhance tissue regeneration and reduce fibrosis. The advancements in the new genomic technologies such as CRISPR-Cas9 have transformed the field of immunology research and will certainly speed up novel discoveries in regulatory and signaling components of inflammation biology.

The newly obtained knowledge will translate into novel therapeutic strategies for inflammatory diseases. For example, recent studies have identified proinflammatory and proregenerative potential of a cytokine IL-22 and a regulatory oncoprotein murine double minute- (MDM-) 2 in the pathogenesis of ischemic renal injury (IRI) and have demonstrated the therapeutic potential of recombinant IL-22 and MDM-2 inhibitor, nutlin-3a, in IRI and other inflammatory diseases [68, 133–135]. Moreover, other studies have identified a pattern recognition molecule PTX3 as a potential target for therapeutic manipulation in damaged tissues as well as a variety of diseases [37, 58]. Therefore, in-depth understanding of the functions of inflammatory cells as well as mediators of inflammation will be instrumental in the identification of novel therapeutic targets and treatment strategies for several inflammatory diseases. As written by a Scottish surgeon in 1974 "Inflammation in itself is not to be considered as a disease but as a salutary operation consequent to some violence or some disease" [136].

Competing Interests

The authors declare no conflict of interests.

Acknowledgments

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Review Article

Interaction between Cannabinoid System and Toll-Like Receptors Controls Inflammation

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Since the discovery of the endocannabinoid system consisting of cannabinoid receptors, endogenous ligands, and biosynthetic and metabolizing enzymes, interest has been renewed in investigating the promise of cannabinoids as therapeutic agents. Abundant evidence indicates that cannabinoids modulate immune responses. An inflammatory response is triggered when innate immune cells receive a danger signal provided by pathogen- or damage-associated molecular patterns engaging pattern-recognition receptors. Toll-like receptor family members are prominent pattern-recognition receptors expressed on innate immune cells. Cannabinoids suppress Toll-like receptor-mediated inflammatory responses. However, the relationship between the endocannabinoid system and innate immune system may not be one-sided. Innate immune cells express cannabinoid receptors and produce endogenous cannabinoids. Hence, innate immune cells may play a role in regulating endocannabinoid homeostasis, and, in turn, the endocannabinoid system modulates local inflammatory responses. Studies designed to probe the interaction between the innate immune system and the endocannabinoid system may identify new potential molecular targets in developing therapeutic strategies for chronic inflammatory diseases. This review discusses the endocannabinoid system and Toll-like receptor family and evaluates the interaction between them.

1. Introduction

Cannabis sativa, better known as marijuana, has been used in traditional medicine for millennia to treat various ailments [1–4]. Development of cannabinoids from the cannabis plant as therapeutic agents has been hindered by their recreational abuse and addictive properties [5, 6]. Legalization of medical marijuana is a growing trend during the past few years. Medical marijuana is primarily used to treat glaucoma and to stimulate appetite and prevent weight loss in AIDS and cancer patients [1–4]. At present, the Food and Drug Administration has approved two cannabinoid medications, Marinol® containing a psychoactive phytocannabinoid and Cesamet® consisting of a synthetic cannabinoid, for the treatment of nausea, emesis, and cachexia [6–8]. In several other countries, Sativex® has been approved to treat spasticity and neuropathic pain in multiple sclerosis patients and pain in patients with advanced cancer [1, 9]. It contains an equimolar combination of psychoactive Δ^9-tetrahydrocannabinol and nonpsychoactive cannabidiol, which are phytocannabinoids [1, 9]. Nevertheless, the beneficial health effects of cannabinoids, for the most part, remain empirical and anecdotal. However, discovery of two major cannabinoid receptors and endogenous cannabinoids in humans provides the opportunity to understand the mechanisms of action and to develop approaches for manipulating the cannabinoid system as effective treatment for particular human diseases.

Familiar effects of marijuana result from phytocannabinoids acting as neurotransmitters or modulating neurotransmitter release that, in turn, causes euphoria, diminished pain, altered sensory perception, impaired memory, and enhanced appetite [6, 7]. Neurological consequences of cannabinoid exposure can be attributed to cannabinoid receptors within the brain. Moreover, cannabinoids impact other biological systems besides the central nervous system. Numerous studies report that cannabinoids suppress *in vitro* functions of human and animal immune cells, and animals exposed to cannabinoids have decreased host resistance to various pathogens and tumors (reviewed in [10–12]). Chronic

cannabis use is associated with increased incidence of rhinitis, pharyngitis, asthma, bronchitis, and sexually transmitted diseases [5, 9, 13, 14]. Besides social behavior contributing to an increased rate of sexually transmitted diseases, depressed immune functions could enhance susceptibility of marijuana users to infections [5].

Because all immune cells examined so far express cannabinoid receptors regardless of their cell lineage, all types of immunity are sensitive to cannabinoid modulation [10–12]. The importance of the cannabinoid system in regulating immune competency is revealed by altered immune status in mice genetically deficient in cannabinoid receptors [15]. In terms of adaptive immunity, cannabinoids usually suppress primary antibody responses to T cell-dependent antigens, induction of cytotoxic $CD8^+$ T cells, and cytokine production by helper $CD4^+$ T cells, whereas other adaptive immune responses are unaffected or enhanced [10–12]. The current view is that cannabinoid exposure skews T cell responses leading to suppression of cell-mediated immunity and inflammatory reactions [10–12]. Furthermore, cannabinoids impact innate immunity that mediates inflammatory responses and promotes initiation of adaptive immune responses. For example, alveolar macrophages isolated from chronic marijuana users have compromised phagocytosis of microorganisms, ability to kill bacteria, and production of proinflammatory cytokines [16, 17]. These consequences of drug use parallel *in vitro* cannabinoid suppression of immune functions by monocytes, macrophages, and macrophage cell lines of human and rodent origins [10–12]. My laboratory reported that cannabinoids impair the ability of murine macrophages to function as antigen-presenting cells resulting in depressed helper $CD4^+$ T cell responses [18–20]. Furthermore, macrophages from mice lacking cannabinoid receptor expression are refractory to cannabinoid suppression of antigen-presenting cell function [21, 22]. Therefore, cannabinoids can exert their influence on an immune response before helper $CD4^+$ T cell activation.

The endocannabinoid system consists not only of cannabinoid receptors, but also of endogenous cannabinoids and their biosynthetic and metabolizing enzymes. Macrophages are major producers of endogenous cannabinoids [23], which may not be a coincidence. Both exogenous and endogenous cannabinoids inhibit proinflammatory cytokine production by macrophages stimulated through Toll-like receptors (TLRs). TLRs play a crucial role in macrophages sensing danger to trigger inflammatory responses. Conversely, activation of macrophages via TLRs alters their expression of cannabinoid receptors and levels of endogenous cannabinoids. This review discusses the endocannabinoid system and TLR family and evaluates the interaction between them with emphasis on the innate immune system.

2. Endocannabinoid System

2.1. Cannabinoid Receptors. Cannabinoid receptors encompass multiple subtypes (reviewed in [24–26]). Central cannabinoid receptor type 1 (CB1) and peripheral cannabinoid receptor type 2 (CB2) are the predominant receptors and share approximately 44% homology [27–30]. Endogenous cannabinoids also bind Transient Receptor Potential Vanilloid 1 receptor, a capsaicin receptor, which is structurally different from CB1 and CB2 receptors [24–26]. The orphan receptor GPR55 may be another receptor subtype, although it has low homology to the other cannabinoid receptors [24–26]. Other candidate receptors have been implicated by pharmacological and functional studies [24–26]. CB1 and CB2 receptors greatly differ in their tissue distribution. CB1 receptor, originally identified in rat cerebral cortex, is primarily expressed in the central nervous system [27, 28]. This receptor subtype is also expressed in various peripheral tissues, such as testis, vascular endothelium, and small intestine [27, 28]. Its expression is heterogeneous within the nervous system and is mainly responsible for cannabinoid psychoactive properties. In contrast, CB2 receptor was originally identified in the promyelocytic leukemic cell line HL60 and is prevalent within the immune system [29, 30]. All lineages of immune cells express the CB2 receptor, although its expression level varies among the cell types. In rank order, B cells express the highest level followed by natural killer cells, macrophages, monocytes, polymorphonuclear cells, and T cells [31]. CB2 receptor expression in healthy brains is limited to a few neurons in the brain stem [32, 33]. However, during neurological diseases, such as multiple sclerosis and Alzheimer's disease, microglial cells, which are brain macrophages, express a high level of CB2 receptor [33, 34]. Some immune cells, including monocytes, also express the CB1 receptor [35–37]. When both receptor subtypes are present in immune cells, the CB2 receptor is usually expressed at a significantly higher level than the CB1 receptor [35–37]. Unlike CB1 receptor-mediated cell activation, signal transduction through the CB2 receptor lacks psychotropic effects [38, 39] making it an attractive target for immunotherapy.

2.2. Exogenous and Endogenous Cannabinoids. Cannabis is a complex mixture of over 100 cannabinoids along with other classes of compounds that have pharmacological and biological activities. Numerous synthetic analogues have been produced based on structure-activity relationship studies [24, 25]. Many synthetic cannabinoid analogues have biological effects similar to their natural counterparts [40, 41]. Cannabimimetic compounds are lipophilic molecules and are classified into four main groups based on their chemical structure [24, 25].

The classical group consists of dibenzopyran derivatives, and the phytocannabinoids fall into this group [24, 25]. Δ^9-Tetrahydrocannabinol is the major psychoactive compound in marijuana and the best-studied cannabinoid. It is a partial agonist binding both CB1 and CB2 receptors and is an ingredient of Marinol and Sativex. Another notable member is cannabidiol that is a nonpsychoactive ingredient of Sativex. Cannabidiol does not activate cannabinoid receptors and yet has biological activity, including immune suppression. Instead, cannabidiol appears to behave as a potent antagonist or inverse agonist [24, 25]. Cannabigerol is the common biosynthetic precursor of Δ^9-tetrahydrocannabinol and cannabidiol [26]. Major

homologues of Δ^9-tetrahydrocannabinol and cannabidiol are Δ^9-tetrahydrocannabivarin and cannabidivarin, respectively, with propyl side chains rather than pentyl side chains [26]. Other members are synthetic analogues of phytocannabinoids, and some synthetic compounds are selective receptor agonists with higher affinity for one or other cannabinoid receptor subtype.

Nonclassical cannabinoids are bicyclic and tricyclic analogues of Δ^9-tetrahydrocannabinol [24, 25]. These synthetic compounds lack a pyran ring. Several are selective cannabinoid receptor agonists. The best-studied member is (−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol referred to as CP55,940, which is a full nonselective agonist with higher potency than Δ^9-tetrahydrocannabinol.

Cannabinoids belonging to the aminoalkylindole group are very structurally different from classical and nonclassical compounds [24, 25]. They were initially developed as potential analogues of nonsteroidal anti-inflammatory drugs [42]. The most widely studied member is (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone called WIN 55,212-2 with slightly higher CB2 than CB1 receptor affinity.

Eicosanoids are the endogenous compounds and are oxidized derivatives of 20-carbon fatty acids [24, 25]. Arachidonoyl-ethanolamide also called anandamide was the first one isolated from porcine brain and was found to have cannabimimetic activity [43]. Anandamide is an arachidonic acid derivative that is highly sensitive to oxidation and hydrolysis. It behaves as a partial agonist and has lower intrinsic activity for CB2 than for CB1 receptor. Another prominent member is 2-arachionoyl glycerol, a monoglyceride, which is more potent than anandamide. Initially, 2-arachionoyl glycerol was isolated from intestine and brain [44, 45], and its concentration in the brain is approximately 170-fold higher than that of anandamide [46]. Because 2-arachionoyl glycerol favors the CB2 receptor, it is viewed as the main endocannabinoid to modulate immune functions. Several synthetic analogues of anandamide have been produced to achieve higher potency and efficacy.

Most cannabinoids exhibit stereoselectivity in pharmacological assays due to chiral centers in the molecules. Frequently, stereoselectivity is also observed in biological assays. Classical and nonclassical cannabinoids and aminoalkylindoles are far more active than their corresponding enantiomer or stereoisomer [24, 25]. Stereoselectivity is one criterion for receptor-mediated actions.

2.3. Cannabinoid Signal Transduction Pathways. Cannabinoid receptors are seven-transmembrane-spanning G protein-coupled receptors [27–30]. The first evidence that cannabinoid receptors are $G_{i/o}$ protein-coupled receptors was cannabinoid-induced inhibition of adenylate cyclase leading to decreased intracellular cAMP level [47]. Pertussis toxin inhibition of a cannabinoid effect confirms $G_{i/o}$ protein-mediated signal transduction [48]. G proteins are heterotrimers, and upon activation the α subunit dissociates from the $\beta\gamma$ dimer (Figure 1). The $G_{i/o}\alpha$ subunit inhibits adenylate cyclase decreasing intracellular cAMP levels [40, 41]. In turn, cAMP-dependent protein kinase A activity diminishes leading to less active transcription factor cAMP response element-binding protein affecting gene expression [40, 41]. The G protein $\beta\gamma$ dimer eventually leads to activation of the mitogen-activated protein kinase (MAPK) pathways and phosphatidylinositol-3 kinase (PI-3K) [49, 50].

MAPK and Akt regulation by cannabinoid receptor signaling pathways is not well understood (Figure 1). PI-3K inhibitors attenuate cannabinoid-induced activation of MAPK in Chinese hamster ovary cells transfected with human CB1 receptor cDNA [50]. This finding indicates that PI-3K leads to Akt activation eventually causing p42/44 and p38 MAPK activation. Similarly, Δ^9-tetrahydrocannabinol activates the PI-3K/Akt pathway in epithelial cells leading to Raf-1-mediated activation of p42/p44 MAPK [41]. In contrast, stimulation of rat microglial cells with 2-arachionoyl glycerol causes MAPK activation that is dependent on protein kinase C, not PI-3K [51]. Conversely, WIN 55,212-2 has the opposite effect and inhibits p42/p44 MAPK activation in murine splenic immune cells [52]. Akt may activate mammalian target of rapamycin (mTOR) present in complex 1 (mTORC1), and, in turn, mTORC1 regulates protein synthesis, glucose metabolism, and autophagy. Rapamycin, a mTOR inhibitor, blocks cannabinoid-induced neural progenitor cell proliferation and cannabinoid-enhanced oligodendrocyte differentiation [53, 54]. On the other hand, WIN 55,212-2 decreases mTORC1 activation in prostate cancer cells [55]. Furthermore, stimulation of promyelocytic HL60 cells with 2-arachionoyl glycerol or other cannabinoid agonists does not activate Akt or mTOR [40]. Thus, cannabinoids may or may not activate Akt, MAPKs, and mTOR depending on the cannabinoid group type, cannabinoid concentration, and/or cell type with differential cannabinoid receptor expression.

While CB1 and CB2 receptors share many signaling steps, distinct differences have been identified in the signaling pathways of theses receptors. CB1 receptor signaling can cause increased intracellular Ca^{+2} level, which may result from phospholipase C activation [40]. Stimulation through the CB1 receptor also activates A-type and inwardly rectifying potassium channels and inhibits N- and P/Q-type calcium currents in neural cells [40, 41, 49]. Most notably, CB1 receptor signaling may increase intracellular cAMP levels and cAMP-dependent protein kinase A activity due to the receptor associating with G_s proteins [40]. Furthermore, the CB1 receptor may associate with G_q proteins leading to phospholipase D activation [40]. While the CB1 receptor may couple to G_s or G_q proteins, the CB2 receptor does not [41].

2.4. Endocannabinoid Biosynthesis and Metabolism. Within the central nervous system, anandamide and 2-arachionoyl glycerol are not preformed molecules stored in vesicles but rather are synthetized when cells are stimulated, such as depolarization of neurons [56, 57]. Their synthesis depends on increased intracellular Ca^{+2} concentration mainly due to Ca^{+2} dependency of the biosynthetic enzymes [58–61].

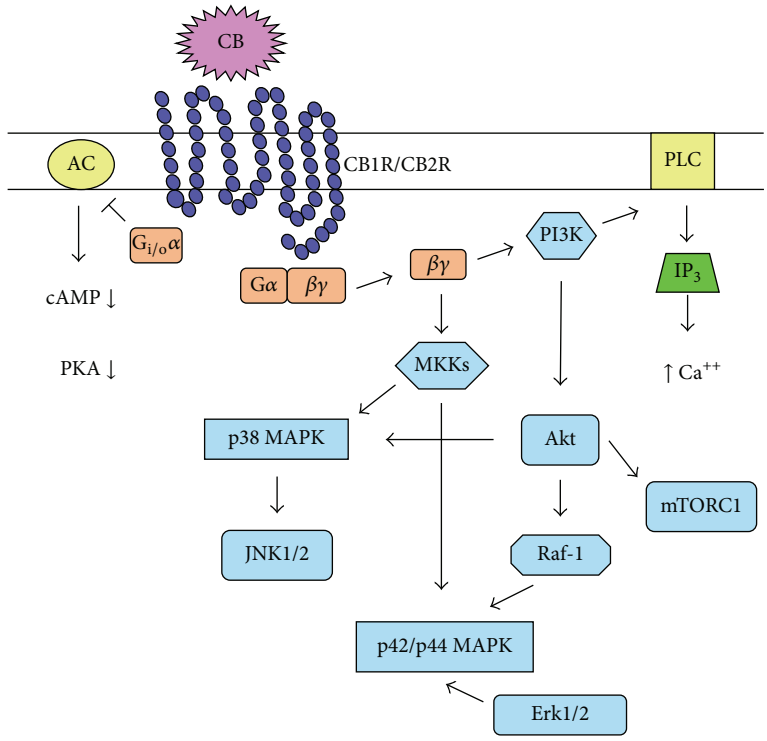

Figure 1: Cannabinoid receptor signal transduction pathway. Upon cannabinoid receptor engagement by a cannabinoid, the activated $G_{i/o}\alpha$ subunit inhibits adenylate cyclase activity causing a decrease in cAMP, which, in turn, decreases PKA activity. The $\beta\gamma$ dimer activates PI3K, which, in turn, activates PLC that ultimately leads to increased intracellular calcium levels. PI3K can active the MAPK pathways. Akt may lead to mTORC1 activation. The $\beta\gamma$ dimer can, also, activate MKK leading to activation of the MAPK pathways. CB: cannabinoid; CB1R: cannabinoid type 1 receptor; CB2R: cannabinoid type 2 receptor; AC: adenylate cyclase; PKA: protein kinase A; PI3K: phosphatidylinositol-3 kinase; PLC: phospholipase C; IP_3: inositol trisphosphate; mTORC1: mammalian target of rapamycin complex 1; MKK: mitogen-activated protein kinase kinases; MAPK: mitogen-activated protein kinase; JNK: Jun kinases; Erk: extracellular signal-regulated kinases.

Anandamide is predominantly produced in a two-step enzymatic process starting with phosphatidylethanolamine that is catalyzed by Ca^{+2}-dependent N-acyltransferase followed by N-acylphosphatidylethanolamine-hydrolyzing phospholipase D (reviewed in [62–65]). The critical precursors of 2-arachionoyl glycerol are *sn*-1-acyl-2-arachidonoylgylerols, and multiple enzymatic pathways generate 2-arachionoyl precursors [63]. The main pathway involves phosphoinositide-selective phospholipase C or similar phospholipases followed by two *sn*-1-selective diacylglycerol lipase isoenzymes (reviewed in [62–65]). In contrast to anandamide, various stimuli in addition to Ca^{+2}-mobilization trigger 2-arachionoyl glycerol synthesis in neural cells, epithelial cells, and macrophages [56, 64]. Unlike anandamide, 2-arachionoyl glycerol is an important precursor of other molecules [63], which may explain their differential resting levels. In most cases, enhanced anandamide and 2-arachionoyl glycerol biosynthesis is limited in time and location, and, thus, their release affects only cells in the nearby vicinity.

After release, endocannabinoids are rapidly internalized into cells by an undefined mechanism, and a proposed transporter has not been definitely identified. Intracellular anandamide is principally hydrolyzed by fatty acid amide hydrolase, an integral plasma membrane protein [66]. Similarly, internalized 2-arachionoyl glycerol is primarily hydrolyzed by monoacylglycerol lipase associated with the plasma membrane [67]. Reaction products do not activate cannabinoid receptors and may recycle back into their respective biosynthetic pathways [63]. One hydrolysis product, arachidonic acid, may be metabolized by various enzymes to produce prostaglandins, thromboxanes, leukotrienes, and other biologically active compounds, which are potent inflammatory mediators or immune suppressors. Anandamide and 2-arachionoyl glycerol themselves may be oxidized by cytochrome P450, cyclooxygenase-2, and 5- and 12-lipoxygenases [63, 65]. Some lipoxygenase products bind both CB1 and CB2 receptors [25, 56, 57]. Hence, results from metabolic enzyme inhibitors or enzyme deficient mice should not presume to be caused by only increased endocannabinoid levels.

3. Toll-Like Receptor Family

A fundamental characteristic of the immune system is the ability to distinguish between self and non-self-molecules or antigens. Immune cells are particularly adept at detecting microbial antigens, and the subsequent immune response can clear an infection and provide protection against a future infection. Innate immunity represents the first line of defense

against infectious diseases, and cells, such as neutrophils and macrophages, are the first responders. Inflammation is the initial immune response against infectious agents, and the inflammatory response promotes initiation of an adaptive immune response by antigen-specific T and B cells. However, cells of innate immunity do not express antigen-specific receptors, unlike T and B cells of adaptive immunity. Decades ago, Janeway proposed a hypothesis that innate immune cells utilize germline-encoded receptors that are antigen-selective [68], and such receptors were eventually identified many years later. Innate immune cells express pattern-recognition receptors that bind conserved molecular patterns, and engagement of these receptors transduces a signal allowing cells to sense danger in the form of a pathogen or host cellular damage [69, 70]. These receptors are present at the cell surface, in endocytic organelles, or in the cytoplasm permitting perception of both extracellular and intracellular dangers. The major receptor gene families based on protein domain homology include TLRs, retinoid acid-inducible gene-1-like receptors, absent in melanoma-2 receptors, C-type lectin receptors, intracellular DNA sensors, and nucleotide-binding domain, leucine-rich repeat-containing receptors (or nucleotide-binding, oligomerization domain-like receptors) that are discussed in several comprehensive reviews [70–76]. This review focuses on the TLR family, which has been extensively investigated and was the first one discovered.

3.1. Toll-Like Receptor Family Members. Toll gene was first identified in *Drosophila* as essential in regulating embryonic development of dorsal-ventral polarity [77, 78]. In addition, Toll has a critical role in resistance to fungal infections in adult flies [79]. This latter discovery was key to understanding innate inflammatory triggers and led to finding the mammalian homologues [80, 81]. Although the number of family members varies among mammalian species, TLRs are evolutionarily conserved type I transmembrane proteins [70–76]. TLRs are predominantly expressed by innate immune cells, especially dendritic cells and macrophages, while adaptive immune B and T cells, and nonimmune cells, including fibroblasts and epithelial cells, have limited TLR expression [70–76]. Family members have a highly conserved cytoplasmic Toll/Interleukin-1 receptor (TIR) domain that initiates signal transduction via recruitment of adaptor proteins [70–76]. At present, 10 TLR chains have been identified in humans, while TLR10 is a pseudogene in mice, but three additional chains (TLR11 to TLR13) are expressed in mice [70–76]. Each TLR recognizes a distinct set of molecular patterns [70–76]. Promiscuous ligand recognition, which is determined by the leucine-rich repeat extracellular domain, is indispensable for the handful of TLRs to mediate effective immune defenses against an array of diverse pathogens.

3.2. Exogenous and Endogenous Ligands. Regarding mammalian host resistance, TLRs bind pathogen-associated molecular patterns (PAMPs) present within microbial molecules, but absent from mammalian molecules [70–75]. The broad gamut of ligand specificity ranges from hydrophobic lipids to hydrophilic nucleic acids. TLRs are divided into two subfamilies based on their cellular location, and the following discussion will focus on the TLR chains expressed in humans. TLR1 and TLR2, TLR4 through TLR6, and TLR10 are plasma membrane proteins, whereas TLR3 and TLR7 through TLR9 reside in membranes of endocytic organelles. Interestingly, ligand recognition correlates with the cellular location of TLRs [70–75]. Cell surface TLRs sense fungal cell wall components (TLR2), bacterial lipopolysaccharides (TLR4), lipoproteins (TLR1, TLR2, and TLR6), flagella (TLR5), or peptidoglycans (TLR2). On the other hand, intracellular TLRs are specific for viral double-stranded RNA (TLR3), single-stranded RNA (TLR7 and TLR8), DNA (TLR9), or unmethylated CpG DNA (TLR9). In contrast, human TLR10 is an orphan receptor without a known ligand. Receptor compartmentalization influences the types of ligands accessible for cell activation. For example, endosomal TLRs engage when virulent intracellular pathogens infect cells, but do not signal when the pathogen remains outside the cell. Although all TLRs possess a similar TIR domain, cell surface and intracellular receptors, in general, utilize different adaptor molecules to transduce a signal [70–75]. Cell surface TLR signaling mainly relies on TIR-containing adaptor protein (TIRAP) and protein myeloid differentiation primary response 88 (MyD88). Furthermore, TIR domain-containing adaptor-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM), also, transmit TLR signals. The different pairs of adaptor molecules activate distinct transcription factors, and, hence, influence the nature and outcome of the inflammatory response.

For many years, one puzzling aspect of innate immunity has been inflammatory responses in the absence of an infection that contribute to tissue damage during autoimmune diseases and chronic inflammatory diseases. Matzinger proposed that the immune system does not distinguish between self and non-self-antigens per se but rather is designed to detect danger [82]. The apparent paradox of sterile inflammation is resolved by realization that pattern-recognition receptors, including TLRs, recognize endogenous ligands. The endogenous molecules called damage-associated molecular patterns (DAMPs) are created or released upon tissue injury or cell death [69, 76]. Heat shock protein 60 was the first candidate DAMP reported to induce TLR4 activation [83]. Within months, necrotic cells were shown to induce proinflammatory gene expression and dendritic cell maturation via TLR2 [84, 85]. Since then, several DAMPs have been identified, and some are intracellular molecules to which innate immune cells are not normally exposed. Analogous to PAMPs, cell surface and intracellular TLRs recognize different types of DAMPs, although endogenous ligands have not been identified for all TLRs [69, 76]. For example, TLR2 and TLR4 ligands include heat shock proteins, serum amyloid A, and oxidized low-density lipoprotein. Intracellular TLR3 recognizes mRNA, and TLR9 binds antibody-chromatin complexes. Recent evidence indicates that danger signals provided by PAMPs and DAMPs synergistically activate inflammatory responses.

3.3. MyD88-Dependent Signal Transduction Pathway. Functional TLRs form dimers and particular receptors associate with a coreceptor and/or an accessory molecule

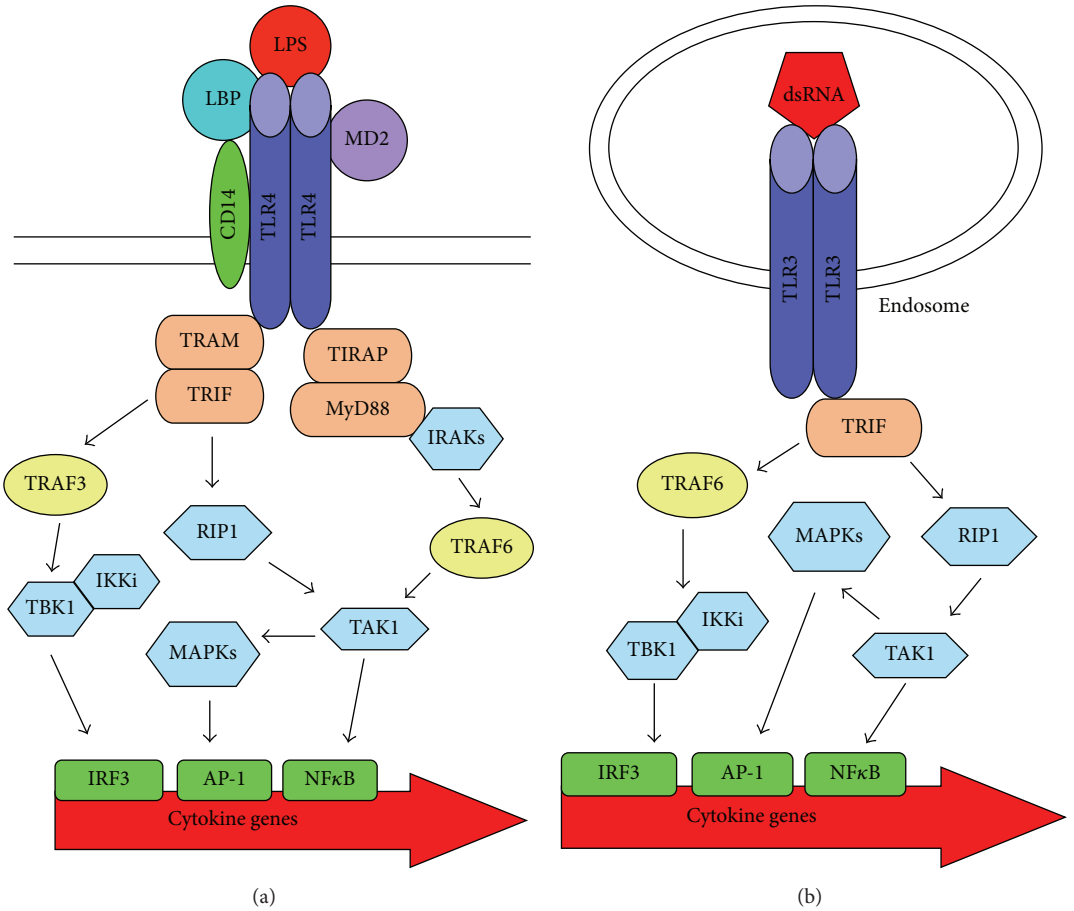

Figure 2: TLR signal transduction pathways. (a) Signaling through TLR4 by bacterial LPS. TLR4 forms a complex with LBP, MD2, and CD14. When LPS binds TLR4, two adaptor complexes are recruited. The MyD88-dependent pathway activates IRAKs eventually leading to activation of TAK1 that leads to activation of transcription factor NF-κB and stimulates the MAPK pathways for transcription factor AP-1 activity. The TRIF-dependent pathway occurs within endosomes and activates RIP1, also resulting in TAK1 activation. Through TRAF3, IKKi and TBK1 are activated leading to the activation of transfer transcription factor IRF-3. (b) Signal through TLR3 by nucleic acids occurs within endosomes. When the receptor is occupied, TLR3 alone binds TRIF without TRAM. RIP1 is activated leading to the same subsequent events as those during TLR4 signaling. TRIF also activates IKKi and TBK1 leading to transcription factor IRF-3 activation, but involves TRAF6, not TRAF3. TLR: Toll-like receptor; LPS: lipopolysaccharide; LBP: LPS-binding protein; MD2: myeloid differentiation-2 protein; MyD88: myeloid differentiation primary response 88; TIR: Toll/interleukin-1 receptor; TIRAP: TIR-containing adaptor protein; TRIF: TIR domain-containing adaptor-inducing interferon-β; TRAM: TRIF-related adaptor molecule; IL: interleukin; IRAK: IL-1R-associated kinases; TNF: tumor necrosis factor; TRAF: TNF receptor-associated factors; TBK1: TANK-binding kinase-1; MAPK: mitogen-activated protein kinase; RIP1: receptor-interacting serine/threonine-protein kinase 1; TAK1: tumor growth factor-β-activated kinase 1; IKKi: inducible IκB kinase-I; IRF-3: interferon regulatory factor-3; AP-1: activator protein-1; NF-κB: nuclear factor-κB.

[69–76, 86]. Most TLRs form homodimers with TLR2 as the notable exception pairing with TLR1 or TLR6. These TLR2 heterodimers have different ligand specificities. When a ligand binds, the TIR domains oligomerize recruiting adaptor proteins that initiate downstream signaling events.

TLR4, the prototype and best-studied receptor, is unique among TLRs using four adaptor molecules. The classic TLR4 ligand is lipopolysaccharide (LPS) from Gram-negative bacteria, which causes septic shock. The LPS response requires a molecular complex (Figure 2(a)) consisting of TLR4 homodimer, LPS-binding protein, CD14, and myeloid differentiation-2 protein (MD-2) [69–76]. MD-2 is required for TLR4 dimerization. Upon LPS exposure, the TLR4:MD-2 complex concentrates within cholesterol-rich lipid rafts containing CD14, which is anchored to the plasma membrane by glycosylphosphatidylinositol. The LPS-binding protein, a soluble serum protein that binds lipid A moiety, initiates sequential transfer of LPS monomers to coreceptor CD14 and then to MD-2. Gioannini and Weiss estimate that LPS monomers extracted from one bacterium are sufficient to activate 1,000 macrophages [87]. Hence, sequential LPS transfer is thought to heighten sensitivity of innate immune cells, in particular macrophages.

Adaptor TIRAP binds the TIR domain of TLR4 but lacks a signaling domain [70–75, 86]. TIRAP itself contains a TIR domain that subsequently recruits adaptor MyD88 to the TLR4 complex (Figure 2(a)). The MyD88-dependent signaling pathway triggers IL-1R-associated kinases and MAPKs

culminating in activation of transcription factors, nuclear factor- (NF-) κB, and activator protein-1. These transcription factors induce nitric oxide and proinflammatory cytokine production.

All TLRs except TLR3 utilize MyD88, and the other cell surface receptors, also, interact with TIRAP similar to TLR4 and lead to NF-κB activation [69–76, 86]. Similar to TLR4, TLR2 heterodimers localize to lipid rafts and require MyD88 via TIRAP for signal transduction. However, TLR2/TLR1 activation involves coreceptor CD14, whereas CD36 serves as the coreceptor for TLR2/6 [70, 88]. CD36 functions as a scavenger receptor in monocytes and macrophages and participates in phagocytosis and endocytosis [89]. CD36 might mediate TLR2/6 internalization to downregulate cell activation.

3.4. TRIF-Dependent Signal Transduction Pathway. Unlike other TLRs, TLR4 signals involve a second pair of adaptor proteins resulting in type I interferon production (Figure 2(a)) and a second wave of NF-κB activation [69–76, 86]. In this pathway, adaptor TRAM and TRIF molecules drive cell activation. Analogous to TIRAP, TRAM lacks a signaling domain and recruits a second adaptor molecule, in this case TRIF, to the plasma membrane. TRIF recruits signaling molecules that, in turn, recruit inducible IκB kinase-i and TANK-binding kinase-1 to the complex. Ultimately, transcription factor interferon regulatory factor- (IRF-) 3 becomes activated leading to type I interferon synthesis, especially interferon-β. Receptor-interacting protein kinase-1 activity eventually leads to TRIF-dependent MAPKs and NF-κB activation.

Both TIRAP and TRAM localize to the plasma membrane; however their binding to TLR4 appears to be mutually exclusive [90]. Colocalization, time kinetics, and endocytosis inhibition studies indicate that TRAM engages after TLR4 internalization [70, 72–75, 86]. A novel model of TLR4 signaling proposes that CD14 mediates trafficking of TLR4 to the endosomes, whereupon the TRIF-dependent pathway is induced [70, 72–75, 86]. Hence, the MyD88-dependent and TRIF-dependent signaling pathways are sequestered from each other, and commence at distinct cellular locations.

3.5. Intracellular TLR Signal Transduction Pathways. Intracellular TLRs stimulate cells by divergent pathways compared with TLR4 [70, 72–75, 86]. Intracellular TLR7 to TLR9 do not utilize the TRAM/TRIF pathway. Instead, engagement of these receptors induces type I interferon production via IRF7 and NF-κB activation in a MyD88-dependent manner. The alternative MyD88 pathway triggers IL-1R-associated kinases to activate transcription factors IRF7 and NF-κB. On the other hand, TLR3 employs the adaptor TRIF protein (Figure 2(b)) but does not need either TRAM or MyD88. The high affinity TIR domain of TLR3 directly binds TRIF in the absence of TRAM. Notably, a point mutation within the TIR domain of TLR3 switches its specificity from TRIF to MyD88 [91]. Similar to TLR4, this TRIF-dependent pathway activates transcription factor IRF3 via TANK-binding kinase-1 inducing robust interferon-β secretion.

Cell activation by TLR signals is tightly regulated on multiple levels. Critical regulatory mechanisms range from TLR trafficking and cleavage to protein modification of signaling molecules. Furthermore, negative regulators are important in preventing autoimmune and inflammatory diseases. TLR recognition of PAMPs is crucial for host resistance to infectious diseases, and individuals with defective TLR responses are immune compromised. On the flip side, abnormal TLR activation by PAMPs, mutations in TLR signaling molecules, and TLR activation by DAMPs are associated with the development and pathogenesis of numerous diseases, including autoimmune diseases, hypersensitivities, chronic inflammatory diseases, cancer, and cardiovascular diseases. Hence, cannabinoid modulation of TLR signal transduction pathways may have beneficial or detrimental consequences on human health.

4. Interaction between Cannabinoid and Toll-Like Receptor Activation

Important innate immune cells expressing TLRs are monocytes, macrophages, microglial cells, and dendritic cells. These myeloid cells are closely related. Monocytes circulate in the blood and mature into macrophages or dendritic cells depending on the stimulus. Microglial cells are resident macrophages in the brain. All these cells express cannabinoid receptors, and cannabinoids influence their immune functions. Of note, alveolar macrophages isolated from chronic marijuana users are compromised in secreting proinflammatory cytokines, such as tumor necrosis factor-α and interleukin-6, in response to LPS [16, 17]. This review discusses cannabinoid modulation of TLR ligand responses by innate immune cells, and vice versa.

4.1. Impact of Cannabinoids on Toll-Like Receptor Responses. Cannabinoid studies regarding TLRs have concentrated on bacterial LPS responses via TLR4 as a classic model for inflammation (Table 1). For the most part, exogenous and endogenous cannabinoids interfere with proinflammatory cytokine and nitric oxide production by LPS- or LPS/interferon-γ-stimulated monocytes, macrophages, microglia, and macrophage cell lines in culture [10–12, 92, 93]. However, one study reported increased interleukin-1β secretion by LPS-activated resident peritoneal cells caused by Δ^9-tetrahydrocannabinol [94] in opposition to other investigations [95, 96]. Cannabinoids display biphasic dose-response curves for cytokine secretion in some culture systems [96, 97], which may account for this apparent discrepancy. Interestingly, Δ^9-tetrahydrocannabinol decreases LPS-induced cyclooxygenase-2 expression in mouse macrophage J774 cell line [93], which would diminish endocannabinoid 2-arachidonoyl-glycerol metabolism, thereby augmenting immune suppression. In addition, chronic marijuana use increases CB1 and CB2 receptor expression on peripheral blood monocytes [98], which may enhance cannabinoid sensitivity. Thus, exogenous cannabinoids may alter the endocannabinoid system leading to greater suppression of the LPS response.

Table 1: Cannabinoid effects on *in vitro* TLR responses.

Cells	Stimulus	Effect	Reference
Alveolar macrophages	LPS	↓ inflammatory cytokines	Baldwin et al. [16] Pacifici et al. [17]
Monocytes	LPS	↓ inflammatory cytokines	Zurier et al. [95]
		Impairs differentiation to dendritic cells	Roth et al. [122]
Thioglycollate-elicited macrophages	LPS	↓ tumor necrosis factor-α protein processing	Zheng and Specter [99]
		↑ interleukin-1β	Zhu et al. [94]
	LPS/interferon-γ	↓ nitric oxide synthase-2	Coffey et al. [102]
		↓ nitric oxide	Mestre et al. [92]
		↑ interleukin-10	Correa et al. [109]
Blood mononuclear cells	LPS	↓ interleukin-1α	Watzl et al. [96]
		Biphasic dose-response	Berdyshev et al. [97]
Microglial cells	LPS	↓ cytokine mRNA	Puffenbarger et al. [103]
		↑ IL-1 receptor antagonist	Molina-Holgado et al. [111]
	LPS/interferon-γ	↓ nitric oxide	Merighi et al. [123]
		↑ interleukin-10	Correa et al. [110]
		↓ nitric oxide	Waksman et al. [124]
Dendritic cells	LPS	↓ TLR4 expression	Xu et al. [106]
BV-2 cells	LPS	↓ NF-κB activity	More et al. [101]
		↓ cytokine mRNA	
		↓ interferon-β mRNA	Kozela et al. [125]
J774 cells	LPS	↓ nitric oxide	Chang et al. [93]
		↓ interleukin-6	
		↓ cyclooxygenase-2	
RAW 264.7 cells	LPS	↓ tumor necrosis factor-α protein processing	Fischer-Stenger et al. [100]
		↓ nitric oxide synthase-2	Jeon et al. [104]
		↓ nitric oxide	
		↓ NF-κB activity	
TLR4-transfected HEK293 cells	LPS	↓ interferon-β mRNA	Downer et al. [126]
		↓ IRF3 activity	
Glioma U87MG cells	Peptidoglycan	↓ NF-κB activity	Echigo et al. [105]
TLR3-transfected HEK293 cells	Polyinosinic:polycytidylic acid	↑ interferon-β mRNA	Downer et al. [127]
		↑ IRF3 activity	Downer et al. [126]
		↓ tumor necrosis factor-α	
		↓ NF-κB activity	
Blood mononuclear cells	Polyinosinic:polycytidylic acid	↑ interferon-β mRNA	Downer et al. [126]
		↓ tumor necrosis factor-α	

Cannabinoids directly impair TLR-induced cell activation in culture (Table 1). Δ^9-Tetrahydrocannabinol perturbs posttranslational processing of tumor necrosis factor-α protein in LPS-activated mouse macrophages [99, 100]. Furthermore, cannabinoids diminish proinflammatory cytokine production in LPS-stimulated mouse microglial BV-2 cell line that is accompanied by decreased transcription factor NF-κB activity and corresponding cytokine mRNA levels [101]. LPS stimulates nitric oxide production and release through induction of nitric oxide synthase-2 expression under NF-κB regulation. Similar to cytokine suppression, cannabinoids attenuate nitric oxide release, nitric oxide synthase-2 gene expression and enzymatic activity, and transcription factor NF-κB activation in LPS- or LPS/interferon-γ-activated myeloid cells [92, 102–104]. Analogously, cannabinoids suppress peptidoglycan-stimulated cell growth of a glioma cell

Table 2: Cannabinoid effects on *in vivo* TLR responses.

Animal model	Cell or tissue	Effect	Reference
LPS	Blood & brain	↓ inflammatory cytokines CB1R & CB2R-mediated	Roche et al. [112]
	Cardiovascular system	↓ hypotensive response	Gallily et al. [115]
	Blood	↓ tumor necrosis factor-α ↑ survival	
LPS-induced pulmonary inflammation	Bronchoalveolar fluid	↓ tumor necrosis factor-α	Berdyshev et al. [113]
	Lungs	↓ neutrophil infiltration CB2R-mediated	
C. parvum/LPS	Blood	↓ inflammatory cytokines ↑ survival CB1R-mediated	Smith et al. [114]
D-Galactosamine/LPS	Liver	↓ inflammatory cytokine ↑ interleukin-10 ↓ acute liver failure ↓ cell infiltration CB2R-mediated	Tomar et al. [116]
		↑ survival	Gallily et al. [115]
Sepsis in $CB2R^{-/-}$ mice	Blood	↑ interleukin-6	Tschöp et al. [118]
	Lungs	↑ tissue damage ↓ survival	
$CB2R^{-/-}$ mice		↑ incidence & severity of induced inflammatory various diseases	Reviewed in Buckley [15] & Malfitano et al. [117]
$CB1R/CB2R^{-/-}$ mice		↑ contact dermatitis ↑ delayed-type hypersensitivity	Karsak et al. [119]
		↑ *Influenza*-induced inflammation	Buchweitz et al. [120]
	Alveolar macrophages	More mature phenotype	Karmaus et al. [121]
	Dendritic cells	More mature phenotype	

line via TLR2 with concomitant decreased NF-κB activation [105]. These results indicate that cannabinoids directly interfere with TLR signal transduction.

On the other hand, cannabinoids may also indirectly suppress *in vitro* cytokine production (Table 1). For example, a CB2 receptor-selective agonist prevents LPS-upregulated TLR4 expression on mouse bone marrow dendritic cells rendering the cells less LPS responsive [106], although a similar cannabinoid effect upon TLR4 expression has not been reported for other innate immune cells. Moreover, cannabinoids induce apoptosis that has been proposed as an immunosuppressive mechanism [107, 108]. However, the majority of findings regarding cannabinoid inhibition of TLR-mediated responses cannot be attributed to apoptosis or cell toxicity. Lastly, cannabinoids may induce other immune suppressive processes. Anandamide and a CB2 receptor-selective agonist induce production of interleukin-10, an inhibitory cytokine, in LPS/interferon-γ-activated myeloid cells [109, 110]. CP55,940 induces interleukin-1 receptor antagonist expression in LPS-stimulated microglial cells [111], which interferes with interleukin-1 signals. Thus, multiple modes of action may mediate cannabinoid suppression of *in vitro* LPS responses.

LPS administration in animals is frequently used as *in vivo* models of inflammation and bacterial sepsis (Table 2). A synthetic cannabinoid diminishes LPS-stimulated proinflammatory cytokine levels in the brain and blood of rats [112]. In the LPS-induced pulmonary inflammation model, exogenous and endogenous cannabinoids dose-dependently decrease tumor necrosis factor-α level in bronchoalveolar fluid and reduce neutrophil infiltration into the lungs in mice [113]. Synthetic cannabinoids rescue *C. parvum*-primed mice from LPS lethality and diminish serum proinflammatory cytokine levels [114]. Likewise, a nonpsychoactive synthetic cannabinoid abolishes LPS-induced hypotensive response in rats and rescues mice from the lethal effects of LPS and D-galactosamine coadministration [115]. A CB2 receptor-selective agonist also protects against mortality and acute liver failure, decreases proinflammatory cytokines levels, and increases inhibitory interleukin-10 level in mice given LPS and D-galactosamine [116]. Upon *in vitro* LPS stimulation, cytokine and nitric oxide production by macrophages

from mice previously given Δ^9-tetrahydrocannabinol remain suppressed without additional drug in the cultures [102]. Perhaps, cannabinoid immune suppression induced *in vivo* may persist after drug removal. Therefore, the effects of cannabinoids on LPS activation in several animal models, in general, parallel the *in vitro* findings.

Involvement of cannabinoid receptors in suppressing TLR responses has been established by multiple approaches. One set of criteria is cannabinoid receptor-selective agonists causing inhibition and cannabinoid receptor-selective antagonists reversing inhibition. Cannabinoid suppression of proinflammatory cytokine and nitric oxide production is CB2 receptor-mediated in LPS-stimulated cultured cells, LPS-induced pulmonary inflammation, and LPS/galactosamine-induced acute liver failure [113, 116] (Table 2). On the other hand, protection of *C. parvum*-primed mice from LPS lethality and diminished NF-kB activity in TLR2 ligand-stimulated glioma cells are mediated through the CB1 receptor [105, 114], whereas both cannabinoid receptors participate in decreasing cytokine levels after *in vivo* LPS administration [112]. Mice genetically deficient in cannabinoid receptor expression are the best evidence for receptor participation. CB2 receptor-deficient mice are highly susceptible to induced inflammatory diseases, including contact dermatitis, experimental autoimmune encephalomyelitis, atherosclerosis, and carbon tetrachloride-induced liver damage [15, 117]. Incidence and severity of the diseases are exacerbated in the CB2 receptor-deficient mice compared to wild-type mice [15, 117]. Importantly, the CB2 receptor-deficient mice have lower survival, more pronounced tissue damage, and increased serum interleukin-6 levels in a sepsis model [118]. Mice lacking both cannabinoid receptors have markedly heightened allergic inflammation leading to exacerbated contact dermatitis, delayed-type hypersensitivity, and inflammatory responses to *Influenza* virus [119–121]. Alveolar macrophages and bone marrow dendritic cells from mice deficient in both cannabinoid receptors have a more mature phenotype, have increased expression of major histocompatibility complex class I and class II molecules, and are more efficient in activating T cells [120, 121], suggesting that the absence of endocannabinoid signals may alter differentiation and maturation of innate immune cells towards hyperresponsiveness. Perhaps, the role of the CB1 receptor is more readily detected in *in vivo* models due to interactions among various cell types. The absence of both cannabinoid receptors has a more dramatic impact on the immune system, indicating the possible interplay of cannabinoid receptors during disease processes, which has important implications in developing cannabinoids as therapeutic agents for inflammatory diseases.

Microglial cells play an important role in neuroinflammation and appear to be a special case in terms of cannabinoid receptor-mediated immune suppression. General consensus is that resting microglial cells express a low level of the CB1 receptor and lack CB2 receptor expression [12, 56, 57]. However, microglial cells from diseased tissues or microglial cells activated in culture gain CB2 receptor expression [12, 56, 57]. Criteria used for cannabinoid receptor involvement in diminished proinflammatory cytokine secretion and nitric oxide release from LPS-activated microglial cells in culture reveal all possible outcomes encompassing both cannabinoid receptors, or the CB1, CB2, or no cannabinoid receptor [103, 109, 111, 123–125]. A receptor-independent mechanism despite cannabinoid receptor expression on the cells implies that cannabinoid receptor expression is too low to exert a biological effect, or the receptors are inactive. A receptor-independent mechanism may involve disruption of lipid rafts due to the hydrophobicity of cannabinoids [141]. As discussed above, lipid rafts are critical for proper assembly of the TLR4 and TLR2 complexes, and their disruption would contribute to a decreased TLR response. Differences in agonist or antagonist concentrations, and cell activation state among the studies, may contribute to disparate findings concerning cannabinoid receptor participation. Additional investigation is needed to resolve this issue.

Cannabinoids exert immune suppression when innate immune cells are activated, but not when the cells are resting or quiescent. When direct suppression is cannabinoid receptor-mediated, the two signal transduction pathways (Figures 1 and 2(a)) would cross-talk. Transcription factor NF-κB is activated through the MyD88-dependent signal transduction pathway via both TLR4 and TLR2. Hence, cannabinoids must interfere with the MyD88-dependent signal transduction pathway to decrease NF-κB activity along with diminishing cytokine production and cell growth through TLR4 and TLR2. In some cases, cell permeable cAMP to counteract the active $G_{i/o}$ subunit reverses cannabinoid inhibition of cytokine secretion and nitric oxide release [10–12, 102, 124] suggesting the involvement of decreased protein kinase A activity in mediating suppression. However, protein kinase A activity is not necessary for cytokine gene expression in TLR-activated cells (Figure 2). Other studies suggest cannabinoid regulation of p42/p44 MAPK activation participates in immune suppression [41, 52]. Activation of the MAPK pathways is required for cytokine gene transcription. In this scenario, too much of a positive signal becomes negative. Excessive MAPK activation may generate a negative feedback loop. Although a remaining question is what is the link between the cannabinoid and TLRs signaling pathways, MAPKs are attractive candidates.

Cannabinoids augment interferon-β production during the TLR3 ligand response (Table 1). Very few studies have examined the impact of cannabinoids on TLR3 signal transduction and interferon-β production (Figure 2(b)), and, thus, studies with nonimmune cells are discussed below. Interferon-β is a type I interferon with antiviral and anti-inflammatory activities and is a treatment for multiple sclerosis patients [142]. WIN 55,212-2 does not suppress but rather enhances interferon-β mRNA expression in polyinosinic:polycytidylic acid-activated HEK293 cell line transfected with TLR3 cDNA [127]. Interferon-β transcript upregulation is accompanied by increased MAPK and transcription factor IRF3 activities; however tumor necrosis factor-α secretion and transcription factor NF-κB activity decrease in the TLR3-transfected cells [127]. Analogously, WIN 55,212-2 augments interferon-β mRNA expression but decreases tumor necrosis factor-α secretion in polyinosinic:polycytidylic acid-activated primary astrocytes and peripheral blood mononuclear cells from multiple sclerosis

Table 3: Inflammatory effects on endocannabinoid system.

Cell or tissue	Stimulus *in vitro* or animal model	Effect	Reference
Macrophages	Thioglycollate *in vivo*	↑ CB2 receptor	Carlisle et al. [128]
Microglial cells	Interferon-γ	Induces CB2 receptor	Walter et al. [129] Maresz et al. [130]
Thioglycollate-elicited macrophages	LPS	↓ CB2 receptor	Carlisle et al. [128] Cabral et al. [131]
Microglial cells	LPS *in vivo*	↑ CB2 receptor	Mukhopadhyay et al. [132] Concannon et al. [133]
Blood mononuclear cells	LPS *in vivo*	↓ fatty acid amide hydrolase activity	Wolfson et al. [134]
Spleen & liver	LPS *in vivo*	↓ 2-arachidonyl glycerol hydrolytic activity	Szafran et al. [135]
Monocytes	LPS	↑ anandamide	Varga et al. [136]
Macrophages	LPS	↑ 2-arachidonyl glycerol ↓ 2-arachidonyl glycerol hydrolytic activity	Pestonjamasp and Burstein [137]
Dendritic cells	LPS	↑ 2-arachidonyl glycerol	Matias et al. [138]
RAW 264.7 cells	LPS	Induces CB1 receptor ↑ CB2 receptor ↑ anandamide ↑ N-acyltransferase ↑ phospholipase D	Walter et al. [129] Mukhopadhyay et al. [132] Friedman et al. [139] Liu et al. [23] Pestonjamasp and Burstein [137]
J774 cells	LPS	↑ anandamide ↑ 2-arachidonyl glycerol ↓ 2-arachidonyl glycerol hydrolytic activity	Di Marzo et al. [140] Pestonjamasp and Burstein [137]

patients [126, 127]. Perhaps, excessive MAPK activation leads to decreased NF-κB activity as discussed for the LPS response.The opposing effects on IRF3 and NF-κB activities indicate that the cannabinoid alters a signaling step downstream of TRIF binding TLR3.

In striking contrast, WIN 55,212-2 has the opposite effect on interferon-β mRNA and transcription factor IRF3 in TLR4-transfected HEK293 cells stimulated with LPS [126] (Table 1). Likewise, Δ^9-tetrahydrocannabinol and nonpsychoactive cannabidiol inhibits interferon-β mRNA expression and protein secretion in LPS-activated mouse microglial BV-2 cell line [125]. An antagonist of peroxisome proliferator-activated receptor-α blocks the enhanced interferon-β level without affecting tumor necrosis factor-α level in TLR3-transfected HEK293 cells [127], indicating sensitivity of transcription factor IRF3, but not NF-κB, activity to peroxisome proliferator-activated receptor-α. However, sensitivity of interferon-β inhibition to the peroxisome proliferator-activated receptor-α antagonist was not examined in TLR4-transfected HEK293 cells. The opposing cannabinoid effects on interferon-β induced via TLR3 versus TLR4 signaling pathways (Figure 2) raise the question of why peroxisome proliferator-activated receptor-α does not enhance interferon-β production in LPS-stimulated cells exposed to cannabinoids, unless the receptor is not activated during TLR4 signaling. The disparate cannabinoid effect on interferon-β production implies two different molecular targets in the TLR3 and TLR4 pathways. Perhaps, the TRAM:TRIF and TRIF adaptors, or TRAF3 and TRAF6 used by TLR4 and TLR3, respectively, have differential sensitivity to cannabinoid immune modulation.

4.2. Impact of Toll-Like Receptor Activation on Endocannabinoid System. Cannabinoid receptor expression by immune cells varies depending on the cell type, maturational stage, and activation state. Innate immune cells have a high degree of plasticity, and their cannabinoid receptor expression can be manipulated intentionally (Table 3). Human peripheral blood monocytes, human dendritic cells, and some monocyte/macrophage cell lines constitutively express both CB1 and CB2 receptors [129, 138, 143]. Maturation of human peripheral blood monocytes and monocytic THP-1 cells and differentiation of human promyelocytic HL60 cell line into macrophages by phorbol esters upregulate cannabinoid receptor expression [143–145]. Notably, Δ^9-tetrahydrocannabinol impairs LPS-mediated differentiation of human monocytes into dendritic cells [122]. On the other hand, resident mouse macrophages lack detectable CB1 and

CB2 receptor mRNA, whereas inflammatory thioglycollate-elicited macrophages express a high level of the CB2 receptor, and interferon-γ stimulation may further increase CB2 receptor expression [128]. Microglial cells express no/low level of CB1 receptor mRNA, and CB2 receptor mRNA is undetectable [128, 129, 146]. Stimulation of rodent microglial cells with interferon-γ induces CB2 receptor mRNA expression [129, 130]. The level of cannabinoid receptor expression on immune cells affects cannabinoid immune modulation. For example, a nonpsychoactive cannabinoid inhibits LPS-induced interleukin-6 expression only after human monocytes mature into macrophages [147]. Hence, particular stimuli induce or upregulate cannabinoid receptor expression during myeloid cell differentiation, maturation, and activation affecting their sensitivity to cannabinoids.

The influence of only TLR4 signal transduction has been investigated on the endocannabinoid system in myeloid cells (Table 3). LPS modulation of cannabinoid receptor expression in myeloid cells depends on the experimental system (Figure 3). For example, CB2 receptor mRNA level drops in thioglycollate-elicited macrophages in response to LPS [128, 131]. In contrast, LPS activation of mouse macrophage RAW 264.7 cell line induces CB1 receptor mRNA and upregulates CB2 receptor mRNA expression [129, 132, 139]. Protein kinases A and C inhibitors partially block the LPS effect on CB1 and CB2 receptor expression [132], although involvement of the cAMP-protein kinase A pathway in LPS signaling is controversial [148]. Likewise, *in vivo* LPS administration to rodents upregulates CB2 receptor expression in microglial cells [132, 133]. Importantly, various pathological conditions are associated with altered cannabinoid receptor expression, usually increased CB2 receptor expression [10, 57]. Hence, the differential LPS impact on cannabinoid receptor expression may reflect plasticity of innate immune cells to cues from their microenvironment.

Evidence is growing that endocannabinoid levels change during various disease processes due to altered catalytic activities of the biosynthetic or metabolizing enzymes. In several animal disease models, modified endocannabinoid levels exert pro- or anti-inflammatory effects on innate immune cells based on enzyme inhibitors, transporter inhibitors, and mice genetically deficient in the enzymes. This research area is discussed in detail elsewhere [56, 57, 92, 133, 149].

Danger signals provided to innate immune cells increase endocannabinoid production (Figure 3). LPS administration *in vivo* decreases metabolizing fatty acid amide hydrolase activity in mouse peripheral blood mononuclear cells [134], which would increase anandamide levels (Table 3). Similarly, LPS administration also diminishes 2-arachidonyl glycerol hydrolytic activity within the spleen and liver [135]. Anandamide is barely detected in rat monocytes and macrophage J774 cell line, whereas the cells contain substantial anandamide levels upon LPS stimulation [136, 140]. In addition, LPS activation of rat macrophages and J774 cells also increases 2-arachidonyl glycerol levels due to decreased 2-arachidonyl glycerol hydrolytic activity in the cells [137]. Human immature dendritic cells contain enhanced 2-arachidonyl glycerol, but not anandamide, levels upon LPS activation [138]. Conversely, low dose-LPS

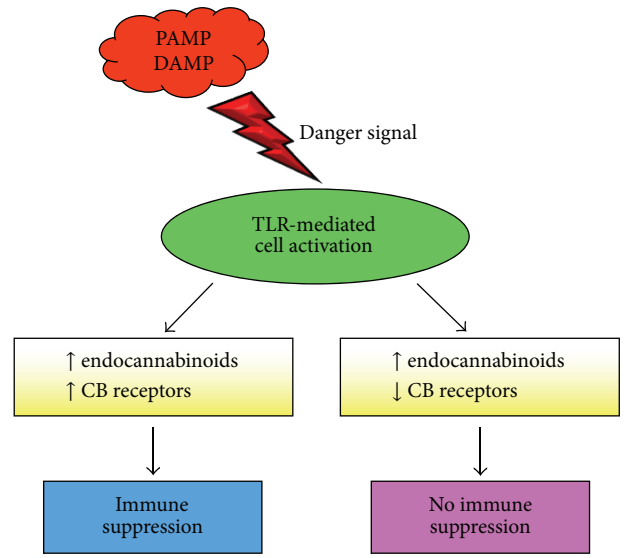

Figure 3: Interaction between TLR-mediated activation and endocannabinoid system. PAMPs and DAMPs provide dangers signals that activate innate immune cells via TLRs. Activated cells increase endocannabinoid levels that may suppress the inflammatory response. Cell activation may increase or decrease cannabinoid receptor expression. Increased receptor expression in the presence of endocannabinoids promotes immune suppression. Decreased receptor expression may render cells resistant to cannabinoid-mediated immune suppression, and the inflammatory response continues. PAMPs: pathogen-associated molecular patterns; DAMPs: damage-associated molecular patterns; TLR: Toll-like receptors; CB: cannabinoid.

activation of macrophage RAW 264.7 cell line increases anandamide, but not 2-arachidonyl glycerol, levels along with enhanced biosynthetic N-acyltransferase and phospholipase D activities [23, 137]. The rapid time kinetics of augmented anandamide level [23] indicates a direct effect of the TLR4 signal transduction pathway as opposed to autocrine stimulation by secreted cytokines. In support of this possibility, increased anandamide level in RAW 264.7 cells is prevented by MAPK and NF-κB inhibitors [23], indicating involvement of the MyD88-dependent signal transduction pathway (Figure 2(a)). Although what endocannabinoids increase during LPS stimulation appears to depend on the cell type and experimental conditions, higher endocannabinoid levels are accompanied by increased biosynthetic enzyme activity and/or decreased metabolizing enzyme activity.

5. Conclusions

When the immune system encounters a pathogen, innate immune cells recognize the pathogen via TLRs and other pattern-recognition receptors to trigger an inflammatory response. Innate immune cells are an important source of endocannabinoids, and these cells synthesize and metabolize endocannabinoids. TLR-mediated activation of the innate immune cells enhances their endocannabinoid levels (Figure 3). In the absence of an infection, tissue damage produces DAMPs perceived as danger signals via TLRs to activate

innate immune cells. Indeed, local endocannabinoid production increases in response to tissue damage during disease progression and infections [150]. Abundant evidence demonstrates that cannabinoids have anti-inflammatory activity, which is the desired consequence during sterile inflammation. Considering that 2-arachidonyl glycerol behaves as a chemoattractant [129, 145], locally enhanced endocannabinoid levels may recruit immune cells to the site and mitigate further tissue damage. Thus, innate immune cells may play a role in regulating endocannabinoid homeostasis, and, in turn, the endocannabinoid system modulates local inflammation. TLR signals also alter cannabinoid receptor expression by innate immune cells, which affects their sensitivity to cannabinoids. During progression of an inflammatory disease, cells may become refractory to cannabinoid immune suppression despite elevated endocannabinoid levels, and the inflammatory response continues and may intensify. Therefore, the final outcome may be enhanced clearance of an infection, facilitation of tissue healing, or exacerbation of tissue damage. Although definition of the link between cannabinoid and TLR signaling pathways awaits further studies, identification of promising molecular targets may provide insights into therapeutic modalities to control injurious inflammation.

Competing Interests

The author declares that there are no competing interests regarding the publication of this paper.

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Review Article

When Insult Is Added to Injury: Cross Talk between ILCs and Intestinal Epithelium in IBD

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Inflammatory bowel disease (IBD) is characterized by an impairment of the integrity of the mucosal epithelial barrier, which causes exacerbated inflammation of the intestine. The intestinal barrier is formed by different specialized epithelial cells, which separate the intestinal lumen from the lamina propria. In addition to its crucial role in protecting the body from invading pathogens, the intestinal epithelium contributes to intestinal homeostasis by its biochemical properties and communication to underlying immune cells. Innate lymphoid cells (ILCs) are a recently described population of lymphocytes that have been implicated in both mucosal homeostasis and inflammation. Recent findings indicate a critical feedback loop in which damaged epithelium activates these innate immune cells to restore epithelial barrier function. This review will focus on the signalling pathways between damaged epithelium and ILCs involved in repair of the epithelial barrier and tissue homeostasis and the relationship of these processes with the control of IBD.

1. Introduction

Inflammatory bowel disease (IBD) comprises a group of intestinal inflammatory conditions that are characterized by an exacerbated chronic inflammation of the gastrointestinal tract. An estimated 1,171,000 Americans are currently diagnosed with IBD and there is increasing prevalence in different regions around the world [1, 2]. IBD includes two related chronic and relapsing conditions, ulcerative colitis and Crohn's disease, that have a severe impact on quality of life. Symptoms of IBD often include abdominal pain, diarrhea, and rectal bleeding, as well as systemic symptoms of weight loss, fever, and fatigue. Periods of remission, in which patients have no symptoms, are alternated by clinical relapses, during which symptoms worsen. These symptoms are the consequence of a dysregulated immune system, of which the origin remains uncertain. It has been suggested that IBD is caused by an abnormal immune reaction to normal constituents of the mucosal microbiota, but genetics and environmental factors also appear to influence disease risk. In addition, changes in the composition of the intestinal microbiota were found to be important environmental factors in IBD pathology [3]. These factors can induce an overactive immune response that damages the mucosal barrier.

In both Crohn's disease and ulcerative colitis, an impairment of the integrity of the mucosal epithelial barrier is observed. The intestinal epithelium of the colon is essential for the absorption of water, but it also has a crucial role as a protective physical barrier by separating the intestinal lumen from the lamina propria. The pathogenesis of IBD differs between ulcerative colitis and Crohn's disease in location and extent of inflammation. While Crohn's disease affects the small bowel and colon, with discontinuous ulceration and full thickness bowel wall inflammation, ulcerative colitis primarily affects the colon, with a continuous inflammation of the mucosa nearly always involving the rectum. In

Crohn's disease, deep ulcers can cause an infection outside the bowel wall, which can spread to the skin or other organs and which can form inflammatory connections called fistula.

In mice, experimental colitis can be induced under several experimental conditions. For example, mice possessing genetic deficiencies in both TGFβRII and IL-10R2 (dnKO mice) develop a disease similar to human ulcerative colitis [4]. However, colitis can also be induced in mice by exposure to *Helicobacter hepaticus* or *Citrobacter rodentium*, by treatment with anti-CD40 monoclonal antibodies (mAbs), by transfer of $CD4^+$ T cells depleted of regulatory T cells into immune deficient mice, or by chemical damage to the colon epithelium, for example, by treatment with dextran sodium sulfate (DSS). Depending on the protocol and dosage of DSS administration, mice can be studied in either a chronic or relapsing colitis model. Both models have similarities with human ulcerative colitis and seem to be driven predominantly by innate immune cells, as disease can be elicited in RAG deficient mice that lack B and T lymphocytes. The exact mechanism by which DSS induces intestinal inflammation remains unknown, but it has been suggested that DSS induces tight junction (TJ) disruption, supporting the hypothesis that barrier dysfunction can occur prior to the onset of mucosal inflammation [5]. DSS-induced colitis is therefore an appropriate model to study the role of candidate genes in colitis promoted by a loss of the epithelial integrity. Consistent with the importance of barrier function, a recent paper showed that increased paracellular permeability for bacterial products, due to claudin 7 deficiency with resulting effects on tight junctions, played a critical role in initiating colonic inflammation [6]. IBD patients also demonstrated increased paracellular permeability with TJ abnormalities. In addition to increased TJ permeability, alterations such as induced epithelial cell death are associated with IBD, and loss of IEC integrity was seen in IBD patients by confocal endomicroscopy before clinical relapse of the disease [7]. However, it should be noted that healthy relatives from IBD patients also showed increased intestinal permeability [8]. Thus, it is unlikely that moderate barrier defects alone are sufficient to cause IBD.

Epithelial barrier integrity is critical to maintain a balance between a symbiosis with the microbiota on the one hand and preventing pathogenic microbes from entering the body on the other hand. The recognition of pathogen-derived and damage-associated molecules upon disruption of the epithelial lining, for example, recognition via toll-like receptors (TLR) expressed by epithelial cells and innate immune cells residing in the epithelium and lamina propria, initiates the production of inflammatory chemokines and cytokines [9]. This recruits innate and adaptive immune cells, which ideally clear the pathogens that entered the lamina propria and help in restoring the epithelial barrier. In order to enable epithelial barrier restoration and reinstate homeostatic conditions, the immune response needs to be downregulated to avoid excessive and unnecessary tissue damage [10]. However, if the pathogen is not sufficiently cleared, or the immune response is not successfully downregulated, chronic inflammation and tissue damage are the consequence.

Communication between mucosal innate immune cells of the myeloid lineage, such as neutrophils, monocytes, macrophages, and antigen-presenting dendritic cells, and the epithelium plays a critical role in regulating gut homeostasis and intestinal inflammation. The recent discovery of innate lymphoid cells (ILCs) added a new player to the field of immunology. ILCs have been implicated in regulating immune homeostasis and inflammatory responses in several instances, such as allergies and bacterial infections [11–13]. Although the ILC population in the intestine is only a fraction of the total lymphocytes, they can contribute to IBD pathology by producing large amounts of cytokines in response to signals from microbes, myeloid cells, and/or epithelial cells [14–16]. Epithelial damage in IBD leads to an altered interaction with the underlying immune cells. Recent findings indicate a critical feedback loop in which damaged epithelium activates ILCs to restore epithelial barrier function [17–20]. Understanding the cross talk between these innate immune cells and the intestinal epithelium in IBD will provide more insight into dysregulation of intestinal homeostasis and IBD pathogenesis.

2. Epithelial Barrier Dysfunction in IBD

The intestinal epithelium is a monolayer of specialized intestinal epithelial cells (IECs), which differentiate from epithelial stem cells into different cell lineages that form crypt and villi structures, including absorptive enterocytes, enteroendocrine cells, mucus-secreting goblet cells, and tuft cells [21]. IECs proliferate in the crypt and migrate to the apical zone while differentiating; mature enterocytes move up the villi and are constantly shed from the villus tip. The continual turnover of epithelial cells helps to maintain barrier function by replenishing the epithelial layer with fresh cells and thereby enables quick repair.

2.1. Epithelial Injury. Damage of the epithelial barrier could be caused by an infection, an exacerbated immune response to normal constituents of the intestine, or a reaction to noninfectious agents. Proinflammatory cytokines from lamina propria immune cells are produced in response to various signals to fight infections and maintain homeostasis. Key antigen-presenting cells (APCs) such as dendritic cells and macrophages are activated in response to molecules from microbes and produce proinflammatory cytokines such as IL-1β, IL-6, IL-18, and tumor necrosis factor (TNF). Different cytokines contribute to the resistance to infections; however, exacerbated cytokine production can lead to pathology. Excessive production of cytokines can cause epithelial barrier dysfunction by altering tight junction and inducing IEC apoptosis and pathological cell shedding. For example, TNF can induce pathological intestinal epithelial shedding by initiating epithelial apoptosis and IL-6 is able to modulate intestinal epithelial tight junction permeability [22, 23]. The regulation of the rate of proliferation and cell death of IECs usually ensures quick and efficient repair of mechanical damage to the epithelium [24]. However, a recent study showed a previously unidentified epithelial-intrinsic alteration of cell death in tissue from IBD patients. Disease-specific alterations

in IECs with impaired Rho-A signalling in IBD patients were found and impaired Rho-A signalling in mice caused cytoskeletal alterations and altered cell shedding, which in turn led to intestinal inflammation [25].

In addition to dysregulated cytokine production, genetic and environmental factors can contribute to intestinal barrier loss. Two well-known susceptibility genes for IBD development are NOD2/CARD15, which is involved in bacterial peptidoglycan recognition, and ATG16L1, which plays a role in autophagy, a process related to processing and presentation of bacterial components [26–29]. It has been found that risk alleles of NOD2 and ATG16L1 are associated with shifts in microbial compositions, associated with development of IBD [3]. Thus, one of the important environmental factors in IBD pathology is a change in the composition of intestinal microbiota. Environmental triggers for IBD are thought to include stress, smoking, diet, and environmental pollution. The underlying mechanisms and their respective contribution to the development of IBD in some people but not in others remain to be elucidated. While environmental factors are believed to contribute to mucosal permeability in IBD, interestingly, permeability is also increased in a proportion of healthy spouses of CD patients [30]. This suggests that an accumulation of multiple factors is necessary to cause IBD.

2.2. Response after Injury. In response to damage, epithelial cells secrete various signals of danger. Levels of damage-associated molecular patterns (DAMPs) or alarmins, including IL-1α and IL-33, are increased in intestinal tissue from IBD patients [31, 32]. These DAMPs are intracellular proteins under homeostatic conditions but are released upon danger signals, such as tissue damage from different cell types in the intestine, including IECs. Activated IECs can produce various cytokines of the IL-1 family including IL-18, IL-33, and IL-37 [32–34]. Whereas IL-18 induces the production of inflammatory cytokines, IL-37 mainly suppresses mucosal innate immune responses and reduces IL-1β and TNF production. IL-33, by contrast, suppresses Th1-type cytokine production and induces neutrophil influx, although in some cases IL-33 in combination with other cytokines augments cytokine production.

APCs, but also neutrophils and ILCs, are potent producers of IL-22 upon activation [35, 36]. This in turn leads to the activation of IL-22 receptors on IECs and the production of various cytokines, for example, IL-10, and antimicrobial peptides such as RegIII-β and RegIII-γ to eliminate bacterial pathogens [37]. IL-22 has recently been shown to promote intestinal stem cell-mediated epithelial regeneration and therefore is considered crucial for the maintenance of epithelial integrity and regeneration after injury [17]. In general, IECs cytokine production and their response to various cytokines are fundamental to mucosal healing [38]. ILCs are activated by and produce cytokines such as IL-6 and TNF, respectively, that not only have a role in epithelial permeability and IEC apoptosis but also are linked to IBD pathogenesis [39–41]. The epithelial response after injury involves different pro- and anti-inflammatory cytokines; the balance of these cytokines seems to be crucial for immune homeostasis and epithelial integrity in IBD.

2.3. Epithelial Repair. After injury of the intestinal epithelium, a process called epithelial restitution initiates wound healing. Enterocytes surrounding the damaged epithelium can migrate into the lesion in response to various cytokines such as transforming growth factor alpha (TGFα), epidermal growth factor (EGF), IL-1β, and interferon-gamma (IFN-γ), although other pathways that induce epithelial restitution also exist [42]. In response, epithelial-derived secreted proteins, including EGF and TGF-β1, coordinate epithelial repair [43, 44]. Epithelial cells respond to signals such as EGF, IL-6, IL-22, and toll-like receptor (TLR) ligands to initiate cell proliferation and differentiation in order to resurface the injury. IL-6 is a key cytokine involved in intestinal epithelial proliferation and wound repair and inhibition of IL-6 results in impaired wound healing due to decreased epithelial proliferation, but it also induces permeability, as stated above [40]. In addition to IL-6, IL-22 induces proliferation of IECs by activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) [45]. In contrast to IL-6 and IL-22, IL-13 and TNF-α seem to be associated with epithelial barrier dysfunction. A study by Heller et al. showed that lamina propria cells from ulcerative colitis patients produced high levels of IL-13, which was found to affect epithelial apoptosis, tight junctions, and restitution velocity [46]. TNF was found to induce iNOS and thereby activates a p53-dependent pathway of IEC apoptosis in chronic ulcerative colitis [41]. Excessive epithelial apoptosis is obviously linked to barrier dysfunction.

Intestinal epithelial cells sense microbes in the gut lumen by pattern recognition receptors (PRR) that include TLRs and nucleotide oligomerization domain-like receptors (NLRs). Functional pathogen recognition is crucial for the generation of an immune response, but TLR signalling has been also shown to promote restoration of damaged epithelia. For example, TLR9-deficient mice have delayed wound repair and are more susceptible to DSS-induced colitis. TLR9-deficiency led to reduced gene expression of hairy enhancer of split 1, an intestinal progenitor cell differentiation factor, and vascular endothelial growth factor (VEGF), a growth factor important for epithelial cell restitution [47].

Recently, IL-36R signalling in IECs, upon intestinal damage, has been linked to the induction of mucosal healing. Defective IL-36 signalling impaired mucosal wound healing and increased susceptibility to DSS-induced colitis. Scheibe et al. found that IL-36R ligands induced proliferation of IECs and their expression of the antimicrobial protein lipocalin 2 [48]. In addition, a study by Medina-Contreras et al. showed that IL-36R deficient mice did not recover from DSS-induced damage [49].

2.4. Barrier Dysfunction. An alteration of epithelial permeability is associated with a loss of mucosal homeostasis, as an impairment of barrier function may fail to sufficiently block pathogen entry. Consequently, pathogen derived antigens are thought to modify the communication of IECs with resident mucosal immune cells, leading to signals that recruit inflammatory cells that clear invading microbes. Genome-wide association studies (GWAS) have identified many IBD susceptibility gene loci, including different STAT proteins

and IL-2, IFN-γ, and IL-10 [50]. Numerous single nucleotide polymorphisms (SNPs) were identified, in coding and noncoding regions, of which the majority were associated with both ulcerative colitis and Crohn's disease. Another study found that IBD risk loci identified by GWAS colocalized with active DNA regulatory elements in intestinal epithelium and immune cells [51]. This suggests that, in addition to variants in protein coding genes, variants in noncoding DNA regulatory regions that are active in intestinal epithelium and immune cells are potentially involved in the pathogenesis of IBD. Recently, the expression pattern of susceptibility genes was found to be tissue specific, demonstrating how these risk loci can contribute to risk of disease through the modulation of a gene expression [52].

Other studies investigated the level of cytokine expression in tissue from patients and found increased colonic mucosal IL-33 in patients with active ulcerative colitis [32], while IL-22 was found to be increased in active Crohn's disease as a response to damage [53]. Both cytokines may be involved in maintaining epithelial integrity and are key examples of the cross talk between ILCs and epithelial cells in IBD and will be discussed further in this review.

3. ILC Populations and Their Function

ILCs have recently been identified as important populations of innate immune effectors and are distributed throughout both lymphoid and nonlymphoid organs, including barrier tissues. They are noncirculating cells that lack antigen-specific receptors encoded by rearranging gene segments, but they differentiate from a common lymphoid progenitor. ILCs can be categorized into functionally distinct subsets, based on their expression of transcription factors and cytokine production [54, 55]. Group 1 ILCs (ILC1 cells and NK cells) express T-bet and/or eomesodermin and produce IFN-γ and TNF-α and are mainly important for the resistance to intracellular pathogens and the response to tumors. Group 2 ILCs are dependent on the transcription factor GATA-3 and produce cytokines such as IL-4, IL-5, IL-9, and IL-13, essential for immune response to helminths. Group 3 ILCs express retinoic acid receptor- (RAR-) related orphan receptor (RORγt) and produce lymphotoxin $\alpha\beta$ (LT$\alpha 1\beta 2$), granulocyte macrophage-colony stimulating factor (GM-CSF), TNF-α, IFN-γ, IL-17A, IL-17F, and/or IL-22 and are involved in eliminating extracellular pathogens and restoring epithelial integrity. Group 3 ILCs can be subdivided into natural cytotoxicity receptor (NCR) expressing cells, which secrete IL-22, and NCR negative cells, which include lymphoid tissue inducer cells (LTi), that express both IL-22 and IL-17 [36]. All three ILC subsets have been implicated in IBD pathogenesis and its control.

3.1. ILCs in Intestine under Homeostatic Conditions. In both small and large intestine, ILC1 are the most abundant ILC population in the intraepithelial compartment. In the lamina propria, however, ILC2 and ILC3 are the dominant populations in the large and small intestines, respectively. Increased numbers of ILC2s are localized in fat-associated lymphoid clusters in the intestinal mesentery, while ILC3s accumulate in the perifollicular areas of Peyer's patches and near intestinal crypts where they can enter and exit and mobilize elsewhere in the tissue, a process that can be inhibited by blocking GM-CSF [14, 56]. It is still debated in which tissues ILCs are generated from their precursors. It has been suggested that different programs can regulate the migration of ILC subsets to the intestine. A study showed that ILC1 and ILC3 need to undergo a retinoic acid dependent homing receptor switch during their development in gut-associated lymphoid tissue to migrate to intestinal tissue, whereas ILC2 can migrate directly [57]. However, Rudensky's group found that ILCs in the gut can be locally renewed and expanded in response to acute environmental challenges such as helminth infection [58]. This indicates that ILCs may self-renew locally or be generated from precursors in these tissues. It has been suggested that cytokines are responsible for the proliferation and activation of mature ILCs. For instance, ILC2 and ILC3 have been shown to be regulated by IL-7 while a particular role for IL-25 has been discovered in ILC2 homeostasis and function [20, 59]. However, it remains unclear whether cytokines can specifically generate individual ILC subsets.

ILCs are able to respond to signals from the microbiota via cytokine signalling through communication with both epithelial cells and intestinal mononuclear phagocytes. However, direct interaction between ILCs and commensal microbes through TLR activation has also been shown [60]. Stimulation of human RORγt$^+$ ILCs with TLR2 agonists resulted in IL-2 production, which in turn enhanced IL-22 expression, suggesting direct sensing of microbial components by ILCs occurrence. In addition, there is accumulating evidence that ILCs may directly interact with bacterial components by NCRs such as NKp46 [61]. For example, NKp46 and NKp44 have been shown to directly bind to epitopes of *Fusobacterium nucleatum* or BCG, respectively [62].

IECs were recently found to regulate the function of ILC3 cells by NFκB activation [63]. An IEC specific knockout of the intrinsic inhibitor of κB kinase α (IKKα), a critical regulator of NFκB, led to defective ILC3 IL-22 responses, which resulted in overproduction of thymic stromal lymphopoietin (TSLP) by IECs. Notably, reductions in expression of lymphotoxin beta receptor- (LTβR-) dependent genes were observed in IECs from IEC specific IKKα knockout mice, suggesting that decreased NFκB activation downstream of LTβR signalling contributed to the impaired IL-22 response.

3.2. ILCs in Intestinal Inflammation. ILCs are able to produce large amounts of cytokines that are linked to IBD pathogenesis. In Crohn's disease patients, an increase in IL-23 responsive ILCs in the intestine was observed [64]. Also, the frequency of the ILC1 subset was much higher in inflamed intestine of people with Crohn's disease [65]. An excessive cytokine production of ILCs is likely contributing to pathogenesis, either directly acting on cells in close proximity or by the recruitment of inflammatory cells. For example, GM-CSF produced by ILCs recruits and maintains intestinal inflammatory monocytes, which can promote inflammation. It has been shown that accumulation of monocytes by GM-CSF derived from NKp46$^+$ ILC3 is sufficient to control

intestinal bacterial infection, even though the capacity to control inflammation is superseded by LTi-like ILC3s and T cells [66].

In general, ILCs are stimulated by cytokines and different cellular stress signals and they can respond with instantaneous cytokine production. For example, ILCs can respond to IL-1 and IL-33 released upon cellular damage and uncontrolled cell death [67, 68]. The various cytokines that different ILC subsets respond to include IL-12, IL-18, IL-1, IL-23, and TNF-family cytokine TL1A [11, 69–71]. Several studies indicate that a combination of cytokines is needed to trigger cytokine production by ILCs [72–74]. Some ILC subsets have been described to modulate adaptive immune responses via MHC class II expression and it has also been suggested that T cells may provide help to ILCs via MHC interactions [13, 75, 76]. Due to their location at the front lines of the mucosa, ILCs are able to quickly react to signals from microbiota, other immune cells in the lamina propria, and epithelial cells. Epithelial-derived signals such as IL-7 are important for the function of ILC2s and ILC3s [59]. Downregulation of epithelial-derived IL-7 expression in mice inhibited inflammation in the gastrointestinal tract of mice in a model of DSS-induced colitis [77]. Emerging studies suggest that ILCs are involved not only in the induction of inflammation but also in the maintenance of the epithelial barrier, in particular with regard to tissue repair and wound healing processes, via the different signalling pathways mentioned below.

4. Cross Talk between Epithelium and ILC

4.1. IL-22/IL-23 Pathway. IL-22 is a key cytokine for epithelial cell mediated immune responses. It is produced by several cell types but principally by ILC3s, and it has been shown to promote intestinal epithelial cell homeostasis and wound healing through STAT3 activation [45]. In addition, interaction of surface LT on ILC3s with LTβR on intestinal epithelial cells can lead to IL-22 production in colon in *C. rodentium* and DSS-induced colitis models [78]. The production of IL-22 by ILCs is induced by IL-23, which is associated with different models of colitis and human IBD [16, 36]. Polymorphisms of interleukin IL-23R have been linked to disease in IBD patients [79]. IL-23R is expressed by various cells, including innate immune cells such as dendritic cells and ILCs but also by epithelial cells. The work of Macho-Fernandez indicated that LTβR signalling in intestinal epithelial cells promotes epithelial IL-23 production in a model of DSS-induced colitis. The authors reported that epithelial-derived IL-23 promoted mucosal wound healing by inducing the IL-22-mediated proliferation and survival of epithelial cells and by increasing mucus production [80]. At steady state, the commensal microbiota stimulated IL-25 secretion by epithelial cells, which inhibited IL-22 production by ILCs [81].

Neutrophils and NK cells are additional important sources of IL-22. Neutrophils and NK cells infiltrate the colonic tissue after DSS-induced injury and produce IL-22 in an IL-23-dependent manner, thereby contributing to immune defense and restitution of epithelial integrity. The transfer of IL-22-competent neutrophils to IL-22 deficient mice even protected the colonic epithelium from DSS-induced damage [82]. Interestingly, in another study, IL-36 expression by neutrophils upon DSS-treatment preceded that of IL-22 and IL-36 shown to play a key role in epithelial barrier repair and resolution of inflammation [49]. It has been shown that maintenance of intestinal stem cells after damage is severely impaired in the absence of ILC3s or the ILC3 signature cytokine IL-22 [18]. In addition, another study found that IL-22 produced by ILCs promotes intestinal stem cell-mediated epithelial regeneration [17]. IL-22 derived from ILC3 cells increased the growth of both mouse and human small intestine organoids by increasing proliferation and promoting intestinal stem cell expansion. Moreover, *in vivo* administration of IL-22 increased epithelial regeneration and reduced intestinal pathology and mortality from induced graft-versus-host disease.

T-bet$^{-/-}$RAG2$^{-/-}$ (TRUC) mice develop spontaneous colitis, which is dependent on IL-23 signalling as well as the presence of neutrophils [74]. In this experimental model of IBD, IL-6 in colonic tissue augmented IL-23 and IL-1α secretion of most likely phagocytes, which in turn stimulated production of IL-17A and IL-22 by NCR^{-} ILC3 [39]. In addition, it has been suggested that IL23R^{+} ILCs can induce colitis via an IL-22-dependent pathway. Neutralization of IL-22 in IL-23R$^{-/WT}$ RAG$^{-/-}$ mice protected the mice from anti-CD40 induced acute colitis. Adding back IL-22 by hydrodynamic injections restored the pathology [15]. In this scenario, IL-22 elevated IFN-γ production, reduced IL-10 levels, and promoted neutrophil recruitment, which is likely the cause for the pathology seen in this model of colitis.

4.2. IL-22/IL-18 Pathway. IL-22 derived from ILC3s can induce IL-18 production in IECs and elevated IL-18 can amplify gut inflammation [83]. In a study by Nowarski et al., deletion of IL-18 or its receptor in intestinal epithelial cells protected mice from DSS-induced colitis and mucosal damage [84]. This group showed that IL-18 inhibited goblet cell maturation by regulating the transcriptional program instructing goblet cell development. These results suggest that goblet cell dysfunction can cause breakdown of barrier integrity and the pathology of ulcerative colitis. Another recent study showed that microbiota-associated metabolites modulated epithelial IL-18 secretion and downstream antimicrobial peptide (AMP) profiles [85]. Hyperactive IL-18 signalling induced colitis by breakdown of the mucosal barrier through induced goblet cell loss. Thus, microbiota-derived metabolites and ILCs can modulate the epithelial expression of IL-18 and thereby regulate the function of the intestinal barrier.

4.3. IL-36 Pathway. Recently, as noted above, it was found that IL-36R signalling is activated upon intestinal damage and stimulates IECs to drive mucosal healing [48]. IL-36 was upregulated in the colonic mucosa of patients with IBD and it was shown that, upon tissue injury, IL-36γ was released from IECs. Impaired proliferation of IECs and delayed wound gap closure were seen in IL-36R knockout mice and, upon mucosal damage induced by DSS, IL-36R ligands activated

colonic fibroblasts that thereupon expressed GM-CSF and IL-6 and induced proliferation of IECs and their expression of the antimicrobial protein lipocalin 2.

In another study, Medina-Contreras et al. showed that IL-36R deficient mice did not recover from DSS-induced damage, which was associated with a reduction in IL-22 expression [49]. Although ILCs are potent producers of IL-22, the decrease of IL-22 expression was only seen in neutrophils. Strikingly, an aryl hydrocarbon receptor agonist increased IL-22 expression from ILC3 and CD4 T cells, which promoted full recovery from DSS-induced damage in these mice, showing the importance of IL-22 for resolution from damage. This signalling pathway could provide potential therapeutic targets to induce mucosal healing and restore epithelial integrity in IBD, especially because the expression of IL-36 is increased in patients with IBD [86].

4.4. TNF Pathway. Tumor necrosis factor (TNF or TNF-α) is a well-studied proinflammatory cytokine that is an essential mediator of inflammation in the gut. However, this cytokine is also known to cause intestinal barrier dysfunction. It is mainly produced by monocytes and macrophages and induces an increase in intestinal epithelial tight junction permeability by activating the ERK1-2 pathway [87]. The TNF superfamily of cytokines may be a promising target for IBD therapy because several members are known to play an important role in IBD pathogenesis. While blocking TNF-α helps many patients, it is not efficient for all patients and can also cause significant side effects, including a hampered host defense [88]. It has been shown that Gram-positive commensal bacteria are vital for the induction of DSS colitis in mice by triggering the recruitment of monocytes and macrophages, which strongly express TNF-α during inflammation [89]. Interestingly, a study by Roulis et al. showed that overexpression of TNF-α by IECs was sufficient to induce spontaneous inflammation of the terminal ileum, similar to Crohn's pathology in humans [90].

Depending on the type of stimulus, human ILC3s can switch between IL-22 and proinflammatory TNF production. For example, a combination of IL-1 and IL-23 preferentially induced IL-22 expression while NKp44 engagement with an agonistic antibody switched the cytokine profile of ILC3s to TNF. However, a combined engagement of NKp44 and the cytokine receptors for IL-1, IL-7, and IL-23 resulted in a strong synergistic effect, inducing both IL-22 and TNF [91]. TNF can also augment the IL-17 production induced by IL-23 in ILC3 cells [92].

4.5. IL-17 Pathway. Another cytokine that is produced by ILC3s upon intestinal inflammation and has been shown to be involved in the maintenance of barrier integrity is IL-17A [93]. During *Helicobacter hepaticus*-induced intestinal inflammation, IL-1α promoted the accumulation of IL-17A secreting ILCs [93]. Bacteria-driven colitis was associated with increased IL-17 and IFN-γ production in the colon and stimulation of colonic leukocytes with IL-23 induced IL-17 and IFN-γ production by innate lymphoid cells that accumulated in the inflamed colon [16]. During epithelial injury, IL-17A regulated expression of occludin, a TJ protein, and thereby limited excessive permeability of the epithelial barrier [94]. Although in this study IL-23R$^+$ $\gamma\delta$ T cells and not ILC3s were identified as the main producers of IL-17A in the lamina propria, it is plausible that ILCs also contribute to epithelial integrity via this pathway. In combination with TNF, IL-17 induced CXCL1, CXCL2, and CXCL5 production from intestinal epithelial cells [95, 96]. These chemokines are involved in the recruitment of neutrophils.

4.6. IL-33 Pathway. Until recently, ILC2 cells had not been implicated in intestinal inflammation. ILC2 cells were mainly associated with cutaneous wound healing, in an IL-33 dependent manner, and recently Monticelli et al. discovered that ILC2 cells have a role in the restoration of intestinal integrity after injury [19, 97]. The group showed a feedback loop of cytokine cues from damaged epithelium to activate innate immune cells to express growth factors essential for ILC2-dependent restoration of epithelial barrier function. This protective function of ILC2 cells seemed to be mediated by the IL-33-amphiregulin (AREG) epidermal growth factor receptor (EGFR) signalling pathway [19]. As discussed above, IECs produce IL-33 upon epithelial injury in the intestine. This study showed that, upon epithelial damage, IL-33 stimulated expression of the growth factor AREG by ILC2 cells. Genetic disruption of the endogenous AREG-EGFR pathway exacerbated disease, demonstrating a critical role for AREG-EGFR signalling in limiting inflammation. Another study by Waddell et al. showed that IL-33 can even protect against oxazolone induced colitis [98]. Because in this study it was also found that IL-33 was increased in the colon of patients with active ulcerative colitis, this suggests the IL-33 pathway has potential for the development of novel therapies.

4.7. IL-25 Pathway. Tuft cells, a rare cell type in the intestinal epithelium, were recently shown to promote ILC2 homeostasis and regulate IL-13 production by ILC2s through constitutive production of IL-25 [20, 99, 100]. In mice infected with *Nippostrongylus brasiliensis*, tuft cell-derived IL-25 increased IL-13 production by ILC2s, which in turn led to increased frequencies of tuft and goblet cells supporting helminth clearance [20]. These results indicate a role for tuft cell-derived IL-25 in the physiological host response to helminths and the maintenance of ILC2s in intestinal epithelium. While this feedback loop between tuft cells and ILC2s is not directly implicated in IBD, ILC2s have recently been suggested to play a role in wound healing in lung mucosa and may very well have a similar function in wound healing in the intestinal mucosa [101]. However, further research is needed to clarify the role of ILC2s in mucosal damage of the intestine.

4.8. IFN-γ Pathway. Bacteria-driven and anti-CD40 induced innate colitis are both associated with an increased production of IL-17 and IFN-γ in the colon, shown by Buonocore et al. [16]. Upon stimulation of colonic leukocytes with IL-23, IL-17 and IFN-γ were produced exclusively by ILCs that accumulated in the inflamed colon [16]. IFN-γ can be produced by both group 1 ILCs and group 3 ILCs.

After injury of the intestinal epithelium, enterocytes surrounding damaged epithelium can migrate into the lesion

in response to various cytokines to initiate wound healing. It has been shown that IFN-γ inhibits enterocyte migration by preventing interenterocyte gap junction communication [102]. Furthermore, IFN-γ has also been identified as an IBD susceptibility gene locus by GWAS, supporting the inflammatory role of this cytokine [50]. In contrast, previous work from Dignass and Podolsky showed that TGFα, EGF, IL-1β, and IFN-γ enhanced epithelial cell restitution by 2.3-fold to 5.5-fold [42]. More recently, Muzaki et al. showed that IFN-γ can trigger an early anti-inflammatory response in intestinal epithelial cells, induced by a particular DC subset [103]. Interestingly, in an innate colitis model in which $RAG^{-/-}$ mice were treated with anti-CD40 antibody and IL-22 was found to be pathogenic, the group of Eken et al. showed a significant reduction of IFN-γ levels in the colon after IL-22 neutralization during colitis [15]. As IL-22 was induced in ILC3 by IL-23 signalling, these data suggest that IFN-γ may contribute to IL-23-IL-23R-dependent colitis. A crucial role for IFN-γ in promoting colitis is further corroborated by Uhlig et al., where the authors abrogate the inflammatory response by blocking IFN-γ [104]. However, the exact role of ILC derived IFN-γ in maintaining epithelial barrier integrity and mucosal inflammation remains to be elucidated.

4.9. ILC Depletion. ILC depletion strategies, using anti-Thy1 or anti-CD90 monoclonal antibodies, have been successful in two different experimental models of IBD, *Helicobacter hepaticus*-induced intestinal inflammation and $T\text{-}bet^{-/-}Rag2^{-/-}$ (TRUC) mice that develop spontaneous colitis, respectively [16, 39]. In contrast, there is also evidence that ILC depletion is not beneficial for intestinal homeostasis. A study by Hepworth et al. showed that depletion of MHC class II^{+} ILC3s dysregulated $CD4^{+}$ T cell responses and promoted spontaneous colitis [105].

So far, ILC depletion is only seen in Simian Immunodeficiency Virus infected macaques and HIV infected humans [106, 107]. Similar to IBD, HIV infection can induce breakdown of the intestinal epithelial barrier. Damage of the mucosal epithelium is preceded by acute HIV infection, which leads to rapid depletion of gastrointestinal IL-17 and IL-22 producing $CD4^{+}$ T cells [108]. Because ILCs, not $CD4^{+}$ T cells, are the most rapid producers of IL-17 and IL-22, Kløverpris et al. investigated the role of ILCs in gut epithelial repair during HIV infection [35, 107, 109]. It was shown that ILCs were depleted from the blood during early acute HIV-1 infection and ILC numbers did not recover after resolution of peak viremia. ILC depletion was associated with upregulation of genes associated with cell death and apoptosis and coincided with epithelial barrier breakdown. During the chronic phase of infection, ILC depletion was associated with altered subset composition and increased expression of activation, tissue homing markers, and Fas death receptor. Tonsil- and gut-resident ILCs were not enriched or depleted in chronic HIV-1 infection, suggesting that the depletion of circulating ILCs was due to apoptosis instead of redistribution. These data suggest that ILC apoptosis could be associated with the loss of epithelial barrier integrity.

5. Conclusion

To date, there is no cure for IBD and current treatments focus on blocking the inflammatory response, which can cause significant side effects, including a hampered host defense. Consequently, new therapeutic approaches particularly focus on target molecules with a more limited range of functions, which also should have less side effects. The feedback loop in which damaged epithelium activates ILCs to restore epithelial barrier function is of interest for future therapeutic approaches. One important feedback loop is the IL-22/IL-23 pathway, where IL-23 derived from activated IECs induces the production of IL-22 from ILCs. IL-22 produced by ILC3s promotes intestinal stem cell-mediated epithelial regeneration and wound healing. Recently, it was found that IL-36R signalling stimulates IECs upon intestinal damage and drives mucosal healing in an IL-22 dependent manner. Besides its role in mucosal healing, IL-22 derived from ILC3s can also induce IL-18 production in IECs and thereby amplify gut inflammation. Dependent on the type of stimulus, ILC3s can switch between IL-22 and TNF production. TNF favors IEC apoptosis and correspondingly augments the IL-17 production induced by IL-23 in ILC3s, a mechanism that is also involved in the maintenance of barrier integrity. In addition, ILC2s are involved in barrier integrity by the IL-33-AREG-EGFR signalling pathway. The different signalling pathways between damaged intestinal epithelium and ILCs indicate that feedback loops between these cell types are critical for maintaining epithelial barrier integrity and tissue homeostasis.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

TLR2-Dependent Signaling for IL-15 Production Is Essential for the Homeostasis of Intestinal Intraepithelial Lymphocytes

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TLR2 signaling is related to colitis and involved in regulation of innate immunity in the intestinal tract, but the mechanisms remain unclear. The aim of this study is to investigate how TLR2 affects differentiation of intraepithelial lymphocytes (IELs) and regulates the susceptibility of colitis. IELs were isolated from the small intestine and colon of mice, respectively. The IEL phenotype, activation, and apoptosis were examined using flow cytometry and RT-PCR. IL-15 expression and IEL location were detected through immunohistochemistry. The experimental colitis was induced by administration of dextran sulfate sodium (DSS). We found that the numbers of CD8$\alpha\alpha^+$, CD8$\alpha\beta^+$, and TCR$\gamma\delta^+$ IELs were significantly decreased in TLR2-deficient mice and the residual IELs displayed reduced activation and proliferation and increased apoptosis, accompanied with impaired IL-15 expression by intestinal epithelial cells (IECs). Further study showed that TLR2 signaling maintained the expression of IL-15 in IEC via NF-κB activation. Moreover, TLR2-deficient mice were found to be more susceptible to DSS-induced colitis as shown by the increased severity of colitis. Our results demonstrate that IECs contribute to the maintenance of IELs at least partly via TLR2-dependent IL-15 production, which provides a clue that may link IECs to innate immune protection of the host via IELs.

1. Introduction

Intestinal epithelial cells (IECs) maintain a fundamental immunoregulatory function that influences the development and homeostasis of mucosal immune cells. Interestingly, this single layer is home to an abundant population of intestinal intraepithelial lymphocytes (IELs). Though primarily comprised of CD8$^+$ T cells, the intestinal IELs can be classified into two major subgroups [1]. One group consists of CD4$^+$ or CD8$\alpha\beta^+$TCR$\alpha\beta^+$ IELs, which are known as type a IELs. Type b IELs express $\alpha\beta$TCRs or $\gamma\delta$TCRs with a unique coreceptor, CD8$\alpha\alpha$. About 65–75% of small intestinal IELs are type b IELs (CD8$\alpha\alpha^+$TCR$\alpha\beta^+$ and CD8$\alpha\alpha^+$TCR$\gamma\delta^+$) in mice. Most CD8$\alpha\alpha^+$ IEL precursors go through a thymic stage of development and complete maturation in the intestine [2]. We and others previously reported that intestinal gut-derived cytokines are particularly important for the homeostatic proliferation and survival of these IELs. For example, specific intestinal IL-7 overexpression (IL-7^{vill} mice) significantly increased the number of type a IELs but did not have much effect on type b cell numbers [3]. Mice lacking the IL-15 system, including IL-15$^{-/-}$, IL-15R$\alpha^{-/-}$, and IL-15R$\beta^{-/-}$ mice, showed a severe reduction in CD8$\alpha\alpha^+$TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ IELs [4, 5], combined with previous evidence showing that cytokines of the common γ-chain (γc) family (e.g., IL-7, IL-15) are critical for development of IELs and depend on γc for cellular signaling.

Toll-like receptors (TLRs) are the main pattern recognition receptors (PRRs) that can recognize pathogen-associated molecular patterns derived from a diverse collection of microbes. TLRs are inducible or constitutively expressed in different combinations throughout the whole intestinal tract by a wide variety of cell types, including IECs, myofibroblasts, and immune cells [6, 7]. TLR signaling has been shown to be

involved in epithelial cell proliferation, cytokine production, and antimicrobial peptide expression [8]. Of the TLRs, TLR2 is involved in recognizing a wide range of ligands, including peptidoglycan [9], lipoteichoic acid (LTA) [10], and lipoarabinomannan [11]. A major signaling target of the TLR2 is activation of the transcription factor NF-κB, which is shuttled from the cytosol to the nucleus where it initiates expression of pro- and anti-inflammatory cytokines [12]. Indeed, our recent study has shown that the higher level of infection burden in TLR2$^{-/-}$ mice was closely associated with a reduction in proinflammatory cytokines in the liver [13]. Furthermore, polymorphisms in genes encoding TLR2 and NF-κB signaling proteins (NFKB1 and NFKBIA) are associated with risk of inflammatory bowel disease (IBD) [14]. Thus, it is hypothesized that TLR signaling for cytokine expression is involved in maintenance of IELs and protection of intestinal mucosa.

In this report, we found that the numbers of IELs (especially type b IELs) were reduced significantly in TLR2$^{-/-}$ mice and residual IELs displayed reduced activation and proliferation, accompanied with impaired expression of IL-15 in intestinal mucosa. Our results also indicate that deficiency of TLR2 contributes to the high susceptibility of mice to dextran sulfate sodium- (DSS-) induced colitis.

2. Methods

2.1. Animals. TLR2$^{-/-}$ mice (C57BL/6J background) were kindly provided by Wenyue Xu (Department of Pathogenic Biology, Third Military Medical University, Chongqing, China). Specific pathogen-free wild-type (WT) C57BL/6J mice were purchased from the Laboratory Animal Center of Third Military Medical University. Six- to eight-week-old, pathogen-free, male mice weighing 20 ± 3 g were used. Mice were maintained in temperature-, humidity-, and light-controlled conditions and all studies were performed under the guidelines of the Institutional Animal Care and Use Committee of Third Military Medical University. All experimental protocols were approved by this committee. For lipoteichoic acid (LTA, Invivogen, USA) injection, LTA dissolved in PBS (5 μg/200 μL/mouse) was injected intravenously via the tail vein, and the intestinal samples were collected 24 h later.

2.2. DSS-Induced Colitis. Animals received either regular drinking water (control) or drinking water within 3% DSS (molecular weight: 36,000–50,000; MP Biomedicals, Cleveland, USA) for 7 days. Fresh DSS solution was provided every day. The mice were checked each day for morbidity and weight was recorded. Mice were sacrificed ten days after the first DSS administration. The colons were removed and colonic sections were stained with H&E.

2.3. Cell Culture. The human colon adenocarcinoma cell line SW480 was purchased from China Center for Type Culture Collection (Beijing, China). The cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (Gibco, Carlsbad, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). Cells were incubated in a 5% CO_2 humidified incubator at 37°C. The culture medium was changed every two days.

2.4. IEL Preparation. Intestinal IEL isolation was carried out as described previously [15]. Briefly, small intestine included tissues from the duodenal bulb to the ileocecal junction. Mesenteric fat and Peyer's patches were carefully removed. The small intestine or colon was opened longitudinally, washed in an IEL extraction buffer (1 mM EDTA, 1 mM DTT in PBS), and cut into 5 mm pieces. The pieces were stirred in the same buffer at 37°C for 25 min. The tissue suspension was filtered rapidly through a glass wool column to remove debris and centrifuged at 1500 RPM at 4°C for 5 min. Pelleted cells were suspended in 20 mL 40% isotonic Percoll (GE Healthcare Biosciences, Piscataway, USA) and centrifuged at 2200 RPM at 4°C for 22 min. The supernatant was carefully sucked off, leaving about 5 mL of solution (containing cells). And the pellet was resuspended in 2 mL Tris-NH_4Cl (heated to 37°C) for red blood cell lysis. The cells were washed twice and resuspended in RPMI1640. The viability of the IELs exceeded 95%, according to trypan blue exclusion staining.

2.5. Flow Cytometric Analysis. The IELs were fluorescence-labeled with the following antibodies: CD45-PE, CD45-PerCP-Cy5.5, CD8α-APC, CD8β-FITC, CD4-PE, TCRβ-APC, TCR$\gamma\delta$-FITC, and CD69-FITC. All antibodies were obtained from eBioscience (San Diego, USA). 2×10^5 cells were suspended in 50 μL staining buffer (eBioscience, San Diego, USA) with saturating amounts of antibodies and incubated for 30 min at 4°C. For BrdU pulse-chase experiments, BrdU dissolved in sterile isotonic saline (10 mg/mL) was injected twice daily (0.1 mg BrdU/g body wt. per d, i.p.), and IELs were collected 24 h later. For detection of BrdU, cells were stained with FITC-conjugated anti-BrdU (BrdU Flow kit; BD Pharmingen, USA), according to the manufacturer's instructions. The apoptotic ratios for the IELs were measured using Annexin-V-FITC/PI Apoptosis Detection Kit (eBioscience, USA) according to the manufacturer's protocol. Data acquisition and analysis were performed with MoFlow (Beckman Coulter, US) and FlowJo (Three Star, Ashland, USA).

2.6. Histological Examination and Immunohistochemistry. Specimens for histological examination were fixed in 4% paraformaldehyde for 48 h. Representative sections of colon and jejunum were cut and embedded in paraffin. 5 μm sections were then cut and stained with H&E.

The small-intestinal tissue blocks were mounted on glass slides. The tissue sections were deparaffinised and rehydrated with xylene and graded alcohol. The primary antibodies, anti-CD3 (1 : 700; Abcam, Cambridge, MA), anti-IL-15 (1 : 200; Abcam, Cambridge, MA), and purified rabbit IgG (10 mg/mL, negative control) were incubated overnight at 4°C. Subsequently, the sections were incubated for 60 min with biotinylated goat anti-rabbit IgG (1 : 100; Wuhan Boster, China) for 60 min, followed by incubation with streptavidin-enzyme conjugate (Wuhan Boster, China). Finally, the sections were counterstained with haematoxylin. An expert

observer, blinded to the experimental conditions, examined the tissue sections.

2.7. Dual-Luciferase Assay. Dual-luciferase assay for NF-κB was performed as described previously [16]. Briefly, twenty-four hours before transfection, 1×10^5 SW480 cells were seeded per well of a 24-well plate. Each pBIIx-luciferase reporter gene plasmid (100 ng, a gift from Dr. Sankar Ghosh's Lab, Yale University) was cotransfected with TK-RL (10 ng, Promega, USA) for normalization and either 25 nmol/L of TLR2-siRNA (sense: 5′-GCCCUCUCUACAAACUUU-ATT-3′; antisense: 5′-UAAAGUUUGUAGAGAGGGCTT-3′) or negative control oligonucleotide (sense: 5′-UUCUCC-GAACGUGUCACGUTT-3′; antisense: 5′-ACGUGACAC-GUUCGGAGAATT-3′). At 24 h after transfection, the cells were stimulated with TLR2 agonist, LTA (500 ng/mL), for 6 h. Then, cell extracts were obtained using the dual-luciferase assay kit according to the manufacturer's protocol.

2.8. Quantitative Real-Time PCR. Total RNA was extracted from isolated IELs using TRIzol (Invitrogen, Carlsbad, CA). RNA was reverse-transcribed into complementary DNA (cDNA) using a SuperScript First-Strand Synthesis System RT-PCR kit (Invitrogen). This cDNA was used as a template for the amplification of KGF, IL-2, IL-10, RegIIIγ, and β-actin. Quantitative PCR was performed by SYBR Premix Ex TaqTM II (TaKaRa, Japan) using an ABI 7500 (Applied Biosystems, USA). The primers selected are as follows: KGF, F: CGCAAA-TGGATACTGACACG, R: GGGCTGGAACAGTTCACA-CT; IL-2, F: CCTGGAGCAGCTGTTGATGG, R: CAGAAC-ATGCCGCAGAGGTC; IFN-γ, F: TCAAGTGGCATA-GATGTGGAAGAA, R: TGGCTCTGCAGGATTTTCATG; RegIIIγ, F: TTCCTGTCCTCCATGATCAAAA, R: CAT-CCACCTCTGTTGGGTTCA; IL-7, F: TCTGCTGCCTGT-CACATCATCT, R: AAGTTTGGTTCATTATTCGGG; IL-15, F: ATGTTCATCAACACGTCCTGACT, R: GCAGCA-GGTGGAGGTACCTTAA; β-actin, F: CTTCTTTGCAGC-TCCTTCGTT, R: AGGAGTCCTTCTGACCCATTC. The expression of each gene was normalized to β-actin expression in the individual samples.

2.9. Statistical Analysis. All data are expressed as means ± SD. Differences were analyzed by Student's t-test (with 95% confidence interval). P values < 0.05 were considered significant.

3. Results

3.1. TLR2 Deficiency Results in the Loss of IELs. To investigate whether TLR2 signaling has an impact on the homeostasis of IELs, we first examined the numbers of IELs in TLR2$^{-/-}$ mice. The number of IELs was analyzed by flow cytometry. The total number of IELs in TLR2$^{-/-}$ mice was reduced significantly in the small intestine and colon, respectively (Figures 1(a) and 1(b)). Immunohistochemistry was used to detect and localize the IELs, and most of these cells expressed CD3. In situ staining for CD3 confirmed that IELs were tightly interdigitated with IECs at the basolateral face (Figure 1(c)). Consistent with flow cytometry, there was a striking loss of CD3-positive cells in the small intestine in the absence of TLR2 compared with WT mice (Figure 1(c)).

3.2. Type b IELs Are Dramatically Decreased in TLR2$^{-/-}$ Mice. To better understand the changes occurring in the IELs after TLR2 knockout, a phenotype analysis was performed using flow cytometry. Typical results are presented in Figures 2(a) and 2(b), and the absolute numbers of IEL subsets are summarized in Figures 2(c) and 2(d). Analysis of small intestinal IELs in TLR2$^{-/-}$ mice revealed that the unconventional CD8$\alpha\alpha^+$ (approximately 3.3-fold) and TCR$\gamma\delta^+$ (approximately 3.9-fold) IELs were dramatically reduced and the CD8$\alpha\beta^+$ IEL subset was significantly reduced (approximately 1.1-fold). There was no significant difference in the number of CD4$^+$ IELs in small intestine between TLR2$^{-/-}$ and WT mice, although their proportion relatively was increased in TLR2$^{-/-}$ mice. Similar results were also observed in the colon of TLR2$^{-/-}$ mice. However, CD4$^+$ IELs from colon were significantly reduced in TLR2$^{-/-}$ mice. These data thus indicate a critical and selective role for TLR2 in the homeostasis of IELs.

3.3. IELs Display Reduced Proliferation and Increased Apoptosis in TLR2$^{-/-}$ Mice. To investigate the contribution of TLR2-dependent proliferative and cell survival signals in IELs, we assessed their proliferative capacity by incorporation of BrdU in vivo. CD8$\alpha\alpha^+$ IELs showed poorer proliferation in TLR2$^{-/-}$ mice, whereas the CD8$\alpha\beta^+$ IELs were normal (Figure 3(b)). We next examined whether TLR2 deletion affected the apoptosis of IELs. The residual IELs showed higher apoptosis in TLR2$^{-/-}$ mice (Figure 3(c)). IELs are T cells that exist in some intermediate stage of activation, and CD69 expression might reflect the activated nature of IELs. Histogram showed a decrement in the expression of CD69 on the IELs from TLR2$^{-/-}$ mice (Figure 3(a)). A panel of cytokines was selected to represent the activities responsible for IEL activation and intestine protection. Results are shown in Figure 3(d). IEL cytokine expressions were significantly altered after TLR2 knockout. The results suggest that IL-2, IFN-γ, and RegIIIγ mRNA expression in TLR2$^{-/-}$ IELs significantly decreased. However, KGF showed no significant difference between WT group and TLR2$^{-/-}$ group (Figure 3(d)).

3.4. TLR2 Signaling in the IECs Maintains IL-15 Expression. We and others have previously reported that IL-7 and IL-15 play critical roles in the development of IELs [17]. We next examined IL-7 and IL-15 mRNA in the mucosa of jejunum by RT-PCR. Interestingly, the basal or LTA-induced expression of IL-15 in mucosa was decreased when TLR2 was absent (Figure 4(a)). However, the defect in IL-7 mRNA expression in TLR2$^{-/-}$ mice was limited (Figure 4(a)). We also used immunohistochemistry to confirm the RT-PCR results suggesting that TLR2-driven signals regulated the expression of IL-15 in intestinal mucosa (Figure 4(b)). It is known that NF-κB binding site is essential for transcriptional

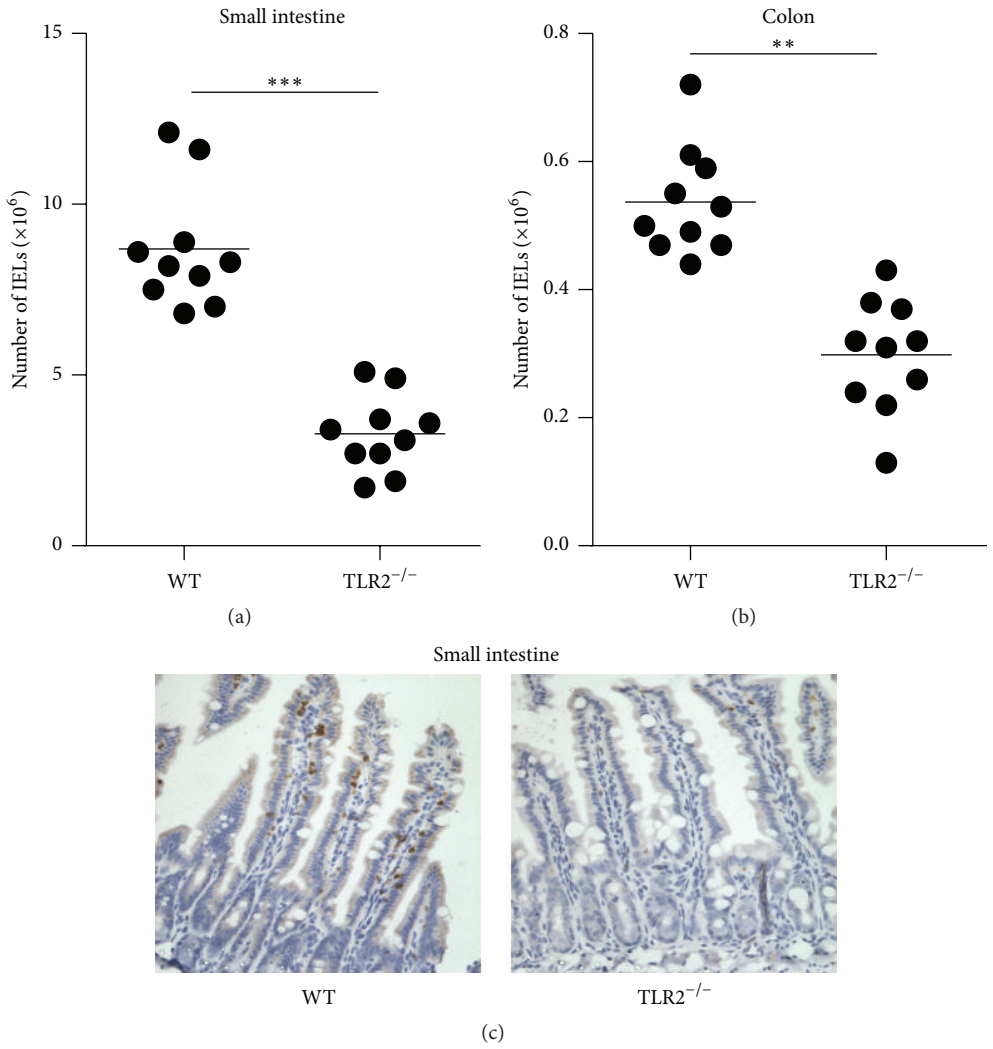


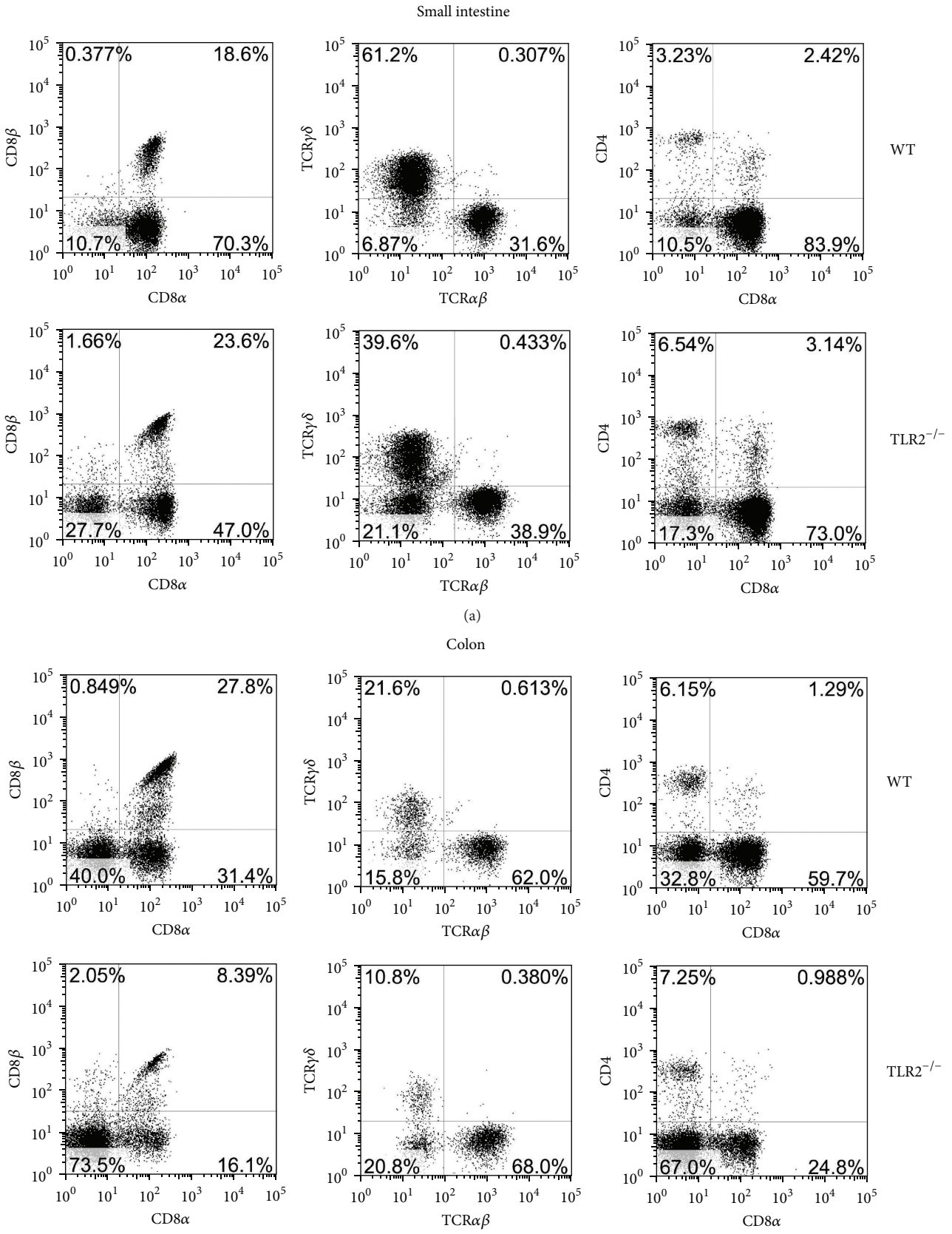
Figure 1: Changes in the number of intraepithelial lymphocytes (IELs) in TLR2$^{-/-}$ mice. (a and b) The numbers of IELs in the small intestine (a) and colon (b) of TLR2$^{-/-}$ mice and individual control mice. Horizontal bars indicate the mean. Ten mice per group from three independent experiments. (c) The IEL was detected through immunohistochemistry with CD3. Images are 400x. $^{**}P < 0.01$; $^{***}P < 0.001$.

activation of the IL-15 gene. To test whether TLR2 agonist could activate signaling pathway in intestinal epithelial cell line, NF-κB reporter activity was assessed after the TLR2 siRNA or mock siRNA-transfected SW480 was stimulated with LTA, respectively. As shown in Figure 4(c), LTA could induce high levels of NF-κB reporter activity, whereas the response of TLR2 siRNA group to LTA was largely suppressed. Thus, our data strongly suggest that TLR2 signaling might maintain the expression of IL-15 in IEC via NF-κB activation.

3.5. TLR2 Deficiency Increases Epithelial Immunopathology. Loss of type b IELs in mice aggravated colitis in several animal models and resulted in impaired ability to repair damaged epithelia. In order to address the consequences of TLR2 deficiency for intestinal physiology, we employed DSS-induced colitis. The severity of the tissue damage in TLR2-deficient colons was illustrated in histological sections, showing diffuse lamina propria and increased destruction of colonic epithelium but reduced immune cell infiltration (Figure 5(a)). The overall mortality rate was 80% in DSS-TLR2$^{-/-}$ mice versus 10% in DSS-WT mice (Figure 5(b)). Compared with WT controls, TLR2 knockout animals lost weight more rapidly, to a greater extent, and failed to gain weight during the course of the 9-day experiment (Figure 5(c)). These data suggest that TLR2 deficiency contributes to the impaired innate immune defense and high susceptibility to colitis in these mice.

4. Discussion

Triggering of PRRs in IECs leads to the expression of immune modulators, which have an impact on the regulation of the adjacent immune cells and are necessary to maintain



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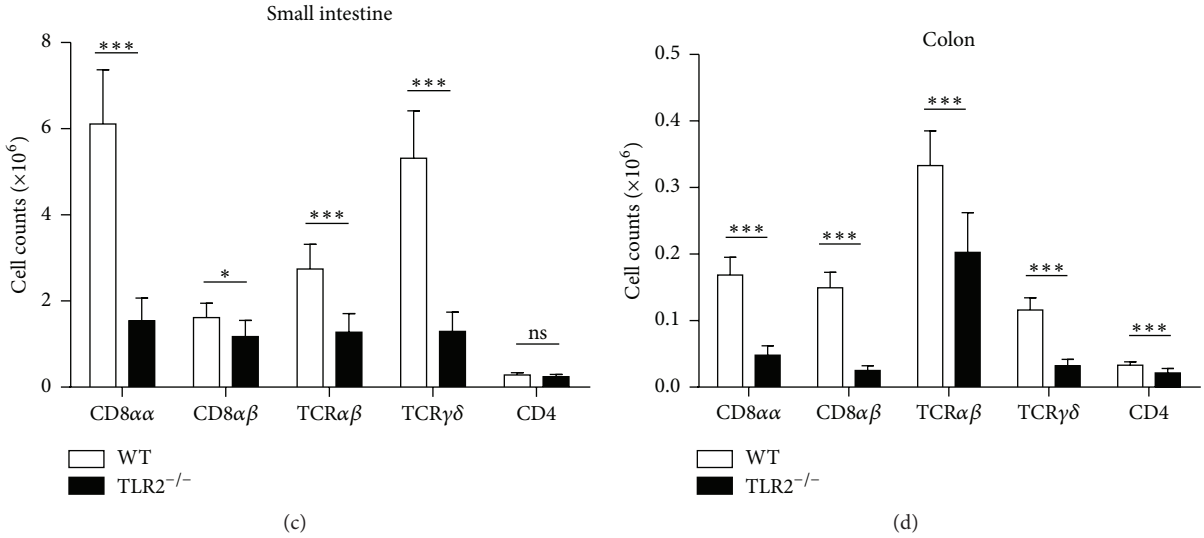
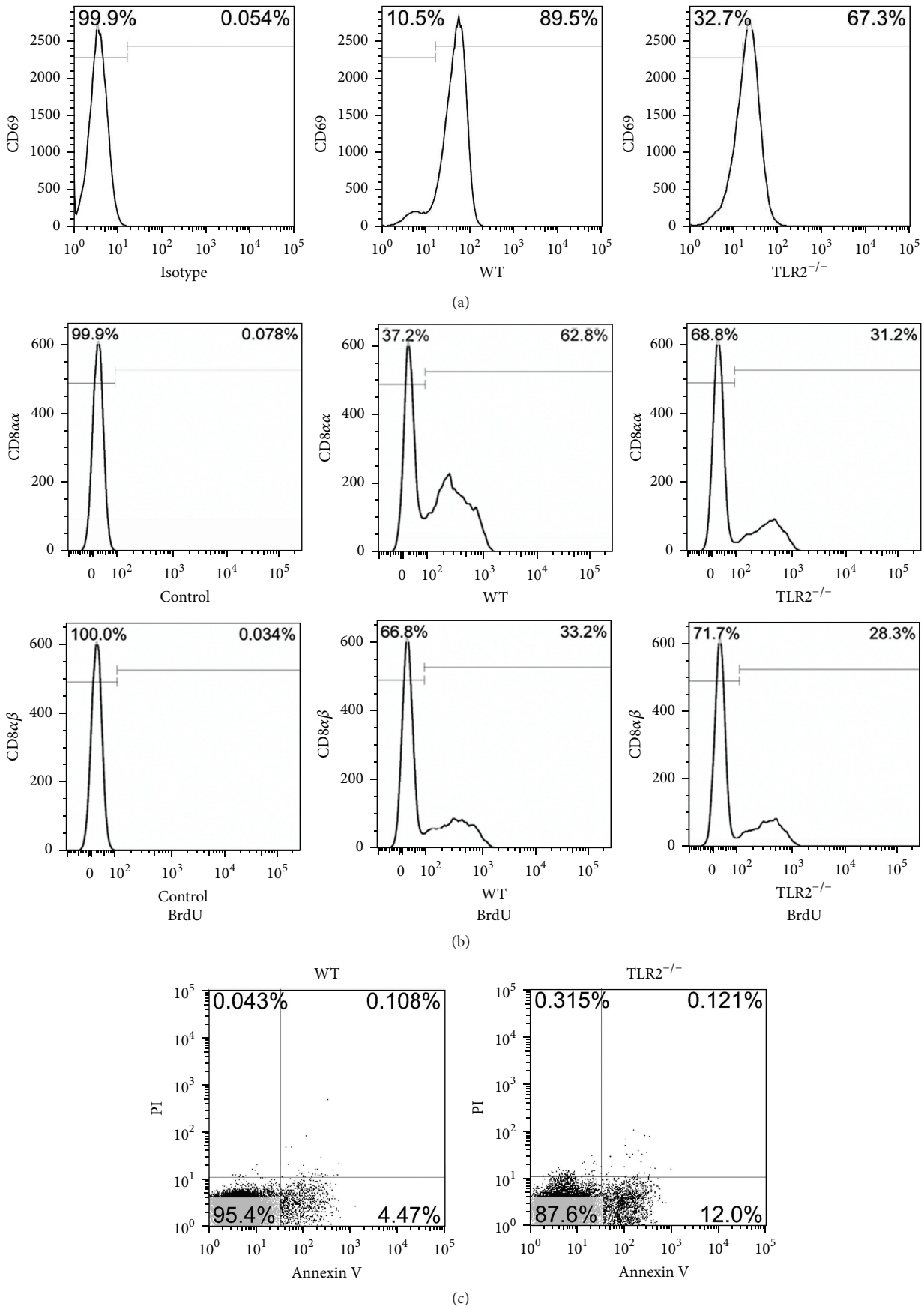


FIGURE 2: Changes in the phenotypes of intraepithelial lymphocyte (IEL) in TLR2$^{-/-}$ mice. Cell populations are expressed as the percentage of gated cells with different cell phenotype markers. The data were obtained from CD45-positive cells. (a and b) The IELs from small intestine (a) or colon (b) of mice were stained as indicated. Expression of CD8α and CD8β chains on CD45$^+$ IELs. Cells were stained with anti-CD45, anti-CD8α, and anti-CD8β mAbs and positively gated by CD45. Expression of TCR$\alpha\beta$ and TCR$\gamma\delta$ on CD45$^+$ IELs. Cells were stained with anti-CD45, anti-TCR$\alpha\beta$, and anti-TCR$\gamma\delta$ mAbs and positively gated by CD45. Expression of CD8α and CD4 on CD45$^+$ IELs. Cells were stained with anti-CD45, anti-CD8α, and anti-CD4 mAbs and positively gated by CD45. (c and d) The absolute numbers of the indicated IEL subsets in the small intestine (c) or colon (d) of individual mice. The absolute number of each subset was calculated by multiplying total number of IELs by the percentage of each subset. Five mice per group. Representative of three experiments. $^*P < 0.05$; $^{***}P < 0.001$.

intestinal mucosa barrier [18]. As IECs have long been recognized as a source of IL-15 in the intestines and are adjacent to IELs, this study determined whether the TLRs on IECs are associated with the homoeostasis of IELs through an IL-15-dependent manner. We found that TLR2-dependent signaling in IECs played an important role in development of IELs (especially CD8$\alpha\alpha^+$TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ IELs), and the signaling was essential in transcriptional activation of IL-15 via NK-κB. Furthermore, we have shown that loss of IELs contributes to the high susceptibility of TLR2$^{-/-}$ mice to DSS-induced colitis.

Although the study of TLR pathways in haematopoietic cells has mostly focused on their proinflammatory properties, their role in maintaining IELs homeostasis and immune tolerance has emerged as a major component of their function in IECs. In the mouse small intestine, the expression of TLR2 is found in 3 IEC lineages: enterocytes, Paneth cells, and enteroendocrine cells [19, 20]. In addition to regional localization, TLR2 expression in the intestine is also thought to be regulated spatially. TLR2 expression is found on both apical and basolateral sides of the follicle-associated epithelium but only the apical side of the villous IECs [7]. Taken together, the localization and expression of TLR2 suggest that the molecular patterns that are actually recognized by TLR2 in the intestine are generally not from pathogens but from the commensal flora. Thus, we focus on the role of TLR2 in shaping the repertoire of IELs in the small intestine and colon. In this study, we showed that the total number of IELs in TLR2$^{-/-}$ mice was reduced significantly in the small intestine and colon as compared with WT mice. Moreover, TLR2$^{-/-}$ mice showed dramatically reduced numbers of CD8$\alpha\alpha^+$TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$, similar to the proportions found in IL-15$^{-/-}$ mice [4]. However, CD8$\alpha\beta^+$ IELs were moderately reduced in TLR2$^{-/-}$ mice. These results suggest that type b IELs are more sensitive to TLR2 deficiency. Interestingly, Shin and Iwasaki found that CD8$\alpha\beta^+$ tissue-resident memory T cells (belong to type a IEL) expressed less IL-15Rb [21]. Based on these facts, we speculated that type b IELs may express more IL-15R compared to type a IELs. Moreover, Yu et al. found that the CD8$\alpha\alpha^+$TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ IELs were selectively decreased in myeloid differentiation factor 88- (MyD88-) deficient mice [22]. And we all know that the MyD88-dependent pathway is utilized by all TLRs except TLR3. Here, we identify previously unappreciated TLR2-mediated signaling necessary for development or maintenance of IEL populations. Consistent with an immune-modulator role of TLR, ~1.8% of all murine transcripts were affected significantly by TLR2 deletion, a number of which correlated with immune processes changes [23]. However, our data showed that the CD8$\alpha\alpha^+$TCR$\alpha\beta^+$ and CD8$\alpha\alpha^+$TCR$\gamma\delta^+$ IEL subsets did not completely disappear in TLR2$^{-/-}$ mice. This may be because the expression of IL-15 in intestinal mucosa was reduced but not completely lost in TLR2$^{-/-}$ mice, which is consistent with our immunohistochemistry findings (Figure 4(b)). Alternatively, the IELs may depend for their development and maintenance on factors other than IL-15, such as IL-2 and IL-7 [24, 25].



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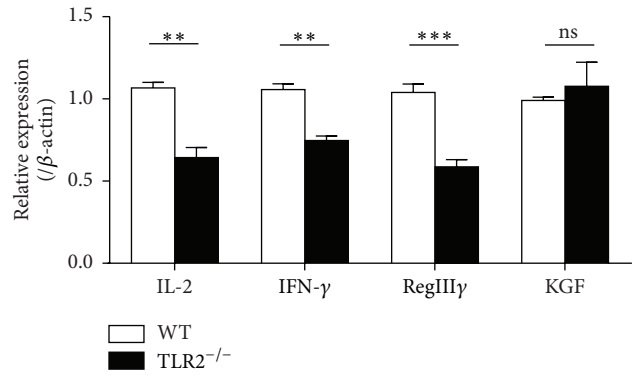

(d)

Figure 3: Alterations in IEL activation, proliferation, apoptosis, and cytokine mRNA expression after TLR2 knockout. (a) The surface expression of CD69 on IELs was detected by flow cytometry. (b) $TLR2^{-/-}$ and wild-type mice were injected with BrdU twice per day. After 24 h, BrdU incorporation in the indicated IEL subsets was analyzed by flow cytometry. (c) Intestinal IELs were examined through flow cytometry for CD45 and apoptosis markers (FITC-Annexin V and PI). In the apoptosis map, FITC-Annexin V+/PI+ indicates late apoptosis, FITC-Annexin V+/PI− indicates early apoptosis, and FITC-Annexin V−/PI− indicates live cells. (d) Changes in the small intestinal IEL-derived cytokine mRNA measured using real-time RT-PCR. The results were expressed as the ratio of the number of copies of the gene of interest to the number of copies of the β-actin gene. Five mice per group. Representative of three experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

Homeostasis of peripheral lymphocytes is regulated by proliferation and apoptosis. Lai et al. reported that IL-15 did not affect IEL development in the thymus but regulated homeostasis of IELs in the intestine [26]. It has been shown that intestinal IL-15 supports $CD8\alpha\alpha^+$ IELs survival through the activation of the PI3K-Akt-ERK pathway to upregulate Bcl-2 and Mcl-1 [27]. In the present study, we showed that the residual IELs displayed increased apoptosis in $TLR2^{-/-}$ mice. In addition to its effects on IEL survival, IL-15 is also known for the ability to enhance proliferation of isolated IELs in vitro [28]. To investigate the contribution of TLR2-dependent proliferative signals in IELs, we assessed their proliferative capacity by incorporation of BrdU in vivo. The $CD8\alpha\alpha^+$ IELs showed poorer proliferation in $TLR2^{-/-}$ mice, whereas the $CD8\alpha\beta^+$ IELs held the normal capacity. Therefore, these results suggest that IEC contributes to the maintenance of IELs at least partly via TLR2-dependent IL-15 production. Unlike T cells in other peripheral immune compartments, IELs express some markers of activated T cells, such as the CT antigen (in mice) and CD69, suggesting that they constitute a large population of "partially activated" effector cells [29]. Moreover, the majority of IELs can secrete epithelial growth factors and produce Th1 cytokines and antimicrobial peptides, such as RegIIIγ [30]. In this study, $TLR2^{-/-}$ mice had a lower level of IEL activation than WT mice. Additionally, the residual IELs in $TLR2^{-/-}$ mice expressed a reduced level of IFN-γ, IL-2, and RegIIIγ. Consistent with our study, other researchers have found that commensal bacteria are required for DSS-induced expression of proinflammatory cytokines and the antibacterial lectin RegIIIγ in IELs [31]. Furthermore, $TCR\gamma\delta^+$ IELs activation was found to be dependent on epithelial cell-intrinsic MyD88 [22]. Thus, the above lines of evidence indicate that IECs supply microbe-dependent cues to IELs via TLRs.

IL-15 was found to be produced only by limited populations of cells, such as dendritic cells and epithelial cells, but not by activated T cells. And perhaps, more importantly, Ma et al. demonstrated that transpresentation of IL-15 by IECs alone is completely sufficient to direct the IL-15-mediated development of $CD8\alpha\alpha^+$ IELs [4]. We thus investigated whether TLR2 signaling affected the production of IL-15 by IECs. Immunohistochemistry results showed that IL-15 is mainly expressed in intestinal epithelium in the basal condition. As expected, the basal expression of IL-15 in epithelium was decreased when TLR2 was absent. It is known that NF-κB binding site is essential for transcriptional activation of the IL-15 gene [32]. In this regard, we observed that the TLR2 siRNA decreased the NF-κB gene reporter activity in response to LTA (TLR2 agonist) in intestinal epithelial cell line SW480. Therefore, as discussed above, it is now apparent that TLR2-MyD88-dependent signaling is important for transcriptional activation of IL-15 gene in IEC and consequently for development and/or maintenance of IEL populations. More researches need to be done for confirming this pathway in IEC and defining the commensal bacterial species that elicit this effect.

Although the etiology of IBD is poorly understood, increasing evidence suggests that IBD is caused in genetically susceptible individuals by a dysregulated mucosal immune response to intestinal microorganisms. Advances risen from genome-wide association studies (GWAS) and immunological studies have recently moved the focus of IBD pathogenesis onto mucosal innate immune responses, such as epithelial barrier integrity, innate microbial sensing, autophagy, and unfolded protein response, as central pathogenic pathways in IBD [33]. IECs from patients with IBD have higher expression of TLRs, especially TLR4, and similar or lower expression of TLR2, TLR3, TLR5, and TLR9 than IECs from control

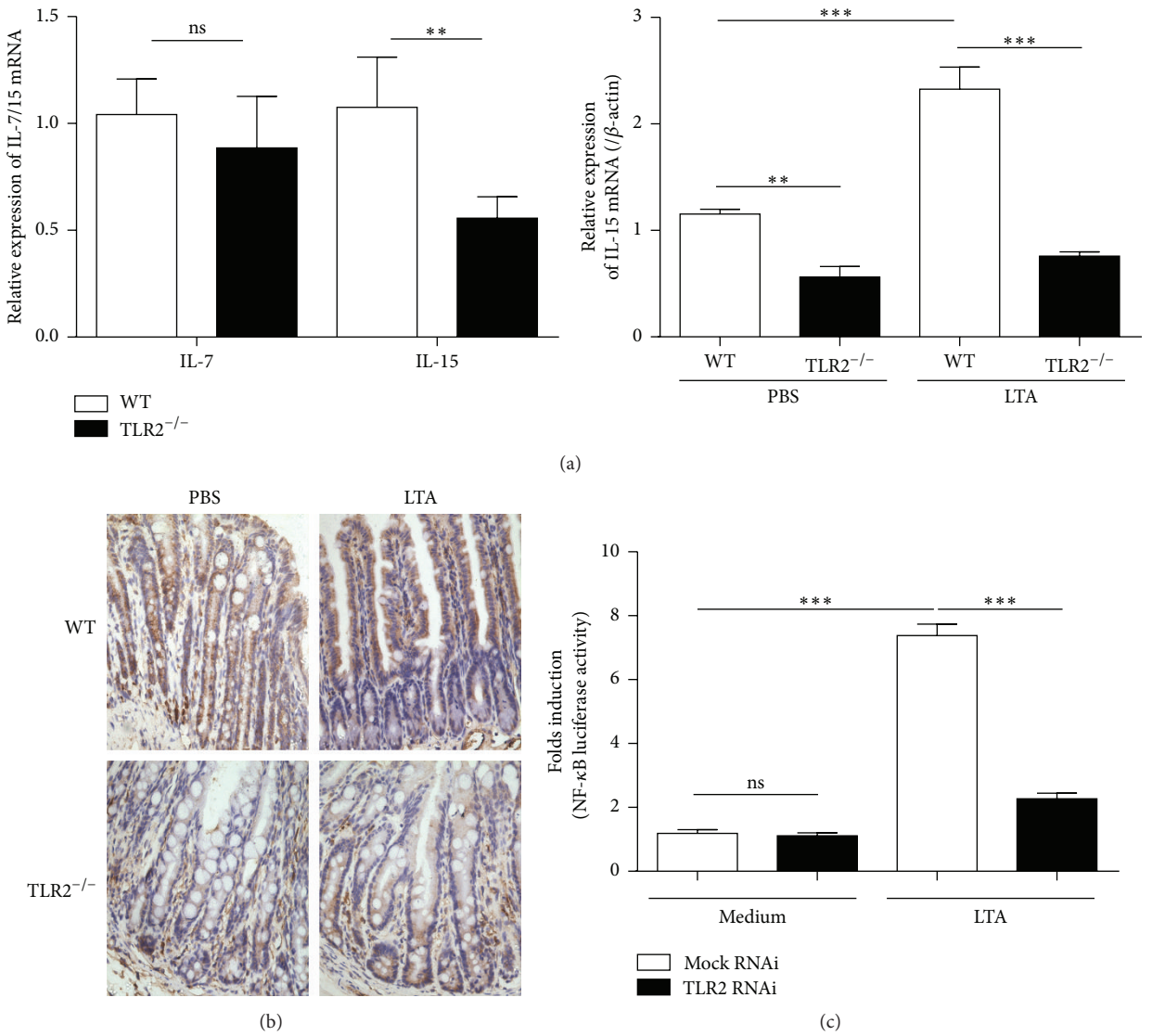

FIGURE 4: The effect of TLR2 knockout (down) on IL-15 expression in intestinal epithelial cells. (a) RT-PCR analysis of IL-7 and IL-15 expression in the jejunum sample. RNA was prepared from the samples of the TLR2$^{-/-}$ mice and the wild-type mice. (b) Immunohistochemistry with IL-15 antibody on sections of small intestine from wild-type mice + PBS, wild-type mice + lipoteichoic acid (LTA), TLR2$^{-/-}$ mice + PBS, and TLR2$^{-/-}$ mice + LTA. Images are 400x. (c) SW480 cells were transfected with TLR2-siRNA or Mock and TK-RL and pBIIx-luc. At 24 h after transfection, the cells were stimulated with the indicated concentrations of LTA, and then both firefly and Renilla luciferase activities were determined using a dual-luciferase assay. The experiments were repeated three times, and all of the data were expressed as the mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$.

individuals [7]. Systemic administration of the TLR2 ligand tripalmitoyl-S-glyceryl cysteine-serine$_4$-lysine (Pam3CSK4) protects against DSS-induced colitis [34]. In this study, TLR2$^{-/-}$ mice were susceptible to the colitis induced by DSS. The above results suggest that IELs dysregulation caused by loss of TLR2 may favor the onset of colitis. Indeed, data from our previous study suggest that loss of CD8$\alpha\alpha^+$TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ aggravates colitis in the mouse model [35]. However, we also cannot ignore the emerging role of TLR2 in protecting TJ-associated integrity and enhancing transepithelial resistance of the enterocyte barrier [34].

5. Conclusion

Collectively, TLR2-dependent signaling is important in keeping the number of the IEL populations in the basal condition. Mice with TLR2 deletion lacked IELs, especially the CD8$\alpha\alpha^+$ IELs (type b IELs), in the small intestine and colon. The residual IELs displayed reduced proliferation and activation and increased apoptosis in TLR2$^{-/-}$ mice, accompanied with impaired IL-15 expression by IEC. Moreover, our results also indicate that deficiency of TLR2 contributes to the high susceptibility of mice to DSS-induced colitis. These results

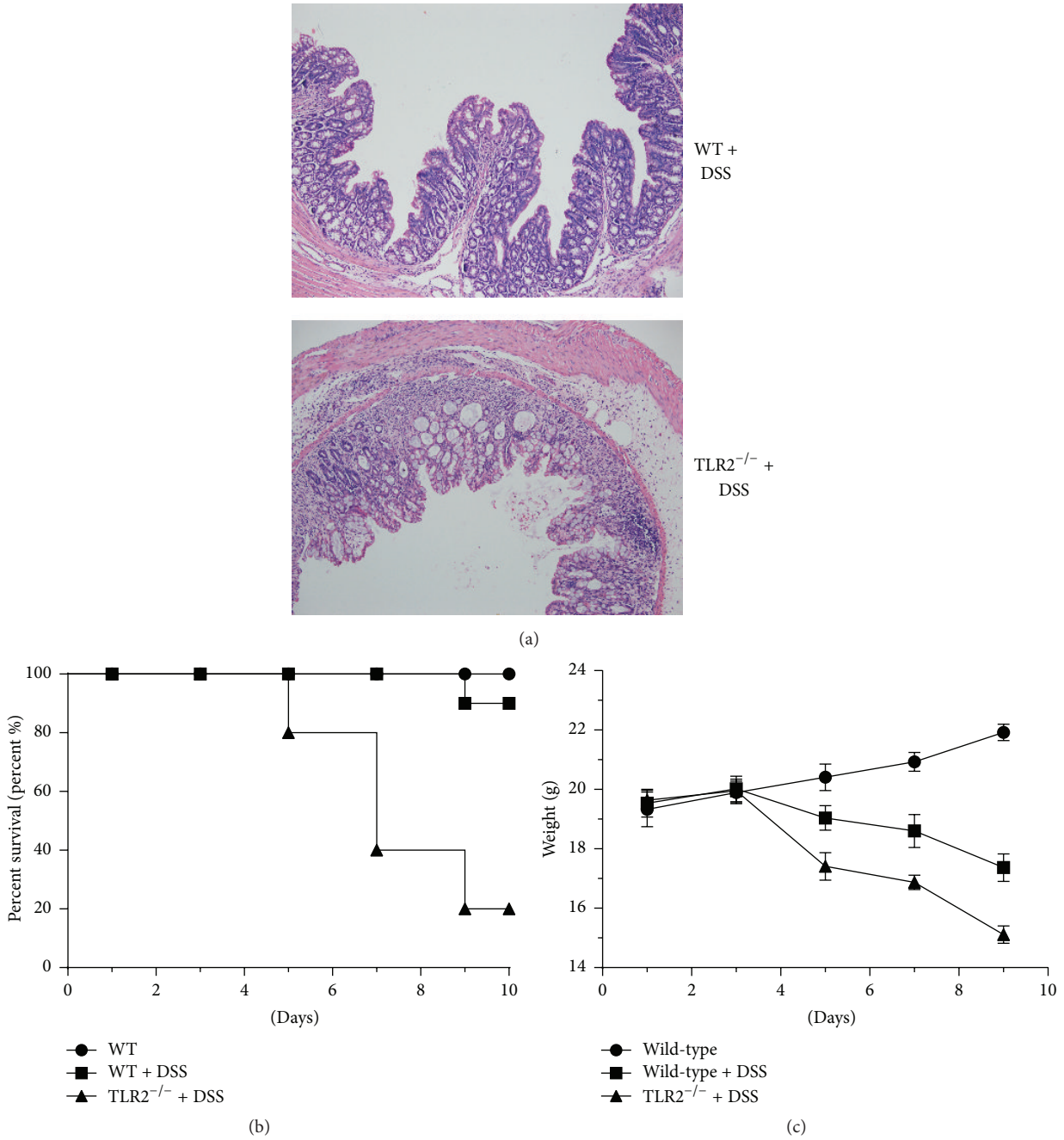

Figure 5: Histopathological characterization of DSS-induced colitis. (a) H&E staining of colons of 3% DSS-treated controls or TLR2-deficient mice. Images are 200x. (b) Survival rates were recorded. (c) Body weight was determined once every other day between day 1 and day 9. Ten mice per group from three independent experiments.

suggest that TLR2-dependent signaling for IL-15 production from interaction between commensal bacteria and IEC plays an important role in maintenance of homeostasis of IEL.

Competing Interests

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

Authors' Contributions

Yuan Qiu and Aimin Pu contributed equally to this work.

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Research Article

Low-Dose Steroid Therapy Is Associated with Decreased IL-12 Production in PBMCs of Severe Septic Patients

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Background. Sepsis-induced immunosuppression may result in higher mortality rates in patients. *Methods*. We examined the relationship of cytokine responses from stimulated peripheral blood mononuclear cells (PBMCs) and monocyte human leukocyte antigen-DR (HLA-DR) expression (days 1 and 7) with low-dose steroid therapy in 29 septic patients. Patients were treated according to the guidelines. Thirty healthy controls were enrolled for validation. *Results*. Eighteen patients were prescribed low-dose steroids and 11 were not. Interleukin- (IL-) 12 responses in patients without low-dose steroid therapy on days 1 and 7 were higher than those with low-dose steroid therapy. Compared to day 1, IL-12 responses significantly increased on day 7 in patients without low-dose steroid therapy. After regression analysis, the change in the IL-12 response from day 7 to day 1 was found to be independently associated with the low-dose steroid therapy. There was no difference in monocyte HLA-DR expression between patients treated with and without low-dose steroid on day 1 or 7. No change in monocyte HLA-DR expression from day 7 to day 1 was observed in patients with or without low-dose steroid therapy. *Conclusion*. Decreased IL-12 response was associated with the low-dose steroid therapy in PBMCs of septic patients.

1. Introduction

Severe sepsis is characterized by acute release of systemic inflammatory (systemic inflammatory response syndrome [SIRS]) and anti-inflammatory mediators (compensatory anti-inflammatory response syndrome [CARS]) caused by infection [1, 2]. The cytokines associated with SIRS include interleukin- (IL-) 1β, IL-6, IL-12, IL-17, and tumor necrosis factor- (TNF-) α. The cytokines associated with CARS include IL-4, IL-10, and transforming growth factor- (TGF-) β1. This immune imbalance results in multiorgan dysfunctions and eventually death in patients with severe sepsis [3].

Dexamethasone inhibited lipopolysaccharides- (LPS-) stimulated release of TNF-α, IL-6, IL-8, and IL-10 from whole blood of septic patients [4]. Similarly, hydrocortisone decreased IL-1 and IL-6 production from *ex vivo* LPS-stimulated whole blood of septic shock patients [5]. Steroid might affect cytokine production from circulatory immune cells of septic patients.

According to the Surviving Sepsis Campaign (SSC) Guidelines for the management of severe sepsis and septic shock (2008 and 2012), patients with septic shock can be prescribed low-dose steroids if adequate fluid resuscitation and vasopressor therapy are unable to restore hemodynamic stability [6, 7]. It is different from the SSC Guidelines of 2004 version [8]. The major cause is the results of the CORTICUS study [9]. Septic shock patients treated with low-dose steroids could reverse shock at an early stage, but final mortality rate was similar to those without low-dose steroids. Low-dose steroid use may also harm patients with septic shock. Low-dose therapy was associated with an increase in adjusted hospital mortality [10]. The adjusted hospital mortality rate was significantly higher (odds ratio = 1.18, $p < 0.001$) in patients who received low-dose steroids compared with those

who did not. However, a multicenter observation study found that steroid use was associated with low mortality rates in patients with severe sepsis [11]. Thus, the use of low-dose steroids in patients with severe sepsis is still controversial [12].

Serial increase in monocyte human leukocyte antigen-DR (HLA-DR) expression and IL-12 response in stimulated peripheral blood mononuclear cells (PBMCs) are associated with higher survival rate in patients with severe sepsis [13]. Thus, we analyzed our study database to explore the relationship of low-dose steroid treatment with HLA-DR expression and cytokine responses in patients with severe sepsis by repeated detections.

2. Materials and Methods

This study is a post hoc analysis using our previously published study database [13].

2.1. Participants and Definitions. From July 2008 to June 2009, 35 patients who were admitted to a 20-bed intensive care unit (ICU) in a regional teaching referral hospital for severe sepsis were enrolled in this study. Six nonsurvivors died within 7 days and they all received low-dose steroid therapy. The data of these six patients was excluded because of lack of repeated cytokine response results for analysis. The SIRS was defined as two or more of the following criteria: (1) body temperature > 38°C or <36°C; (2) respiratory rate > 20 breaths/min; (3) heart rate > 90 beats/min; and (4) white blood count > 12000/μL or <4000/μL or >10% bands. Sepsis was defined as SIRS according to a confirmed infectious etiology. For validating experimental findings, 22 men and 8 women visiting our health evaluation center for examinations were enrolled as healthy controls with mean age of 60.8 ± 1.9 years old.

Severe sepsis was defined according to the consensus criteria of sepsis with one or more organ dysfunctions such as shock, respiratory failure, acute renal failure, jaundice, and thrombocytopenia [14, 15]. Septic shock was defined as sepsis-induced hypotension unresponsive to fluid resuscitation within 24 hr after admission to ICU. Respiratory failure was defined as ventilation dysfunction requiring invasive ventilator support. Acute renal failure was defined as a rapid increase in creatinine levels (>0.5 mg/dL). Jaundice was defined as hyperbilirubinemia (total bilirubin > 2 mg/dL), whereas thrombocytopenia was defined as a platelet count of <150,000/μL. Disease severity was assessed by the Acute Physiology and Chronic Health Evaluation (APACHE) II score [16].

Standard treatment according to guidelines was provided to all patients [6, 8]. A course of low-dose steroid therapy could be prescribed in septic shock patient with 7 days of intravenous hydrocortisone 50 mg every 6 hours if shock developed within 24 hr after admission to ICU. The Institutional Review Board at Chang Gung Memorial Hospital approved our previous study (96-1465B) and the patients' close family members provided informed consent. Patients who survived longer than 28 days after ICU admission were defined as survivors.

2.2. PBMCs Preparation. Whole blood (10 mL) was obtained from each patient at 08:30 a.m., within 48 h of admission to ICU, and immediately mixed with heparin. The day of first blood sampling was defined as day 1. A second blood sample was obtained on day 7. PBMCs were isolated via differential centrifugation over Ficoll-Plaque (Amersham Biosciences, Uppsala, Sweden) from 8 mL of residual whole blood within 2 h of collection.

2.3. Monocyte HLA-DR Measurement by Flow Cytometry. 2.5×10^5 of PBMCs were suspended in 50 μL of phosphate-buffered saline (PBS) and incubated in the dark for 15 min at room temperature with 20 μL of HLA-DR_{PerCP}, $CD11b_{PE}$, and $CD14_{FITC}$ antibodies (Becton Dickinson, CA, USA). Then, the cells were resuspended in 500 μL of PBS. The monocytes were detected by a three-color flow cytofluorimeter (Beckman Coulter, CA, USA) with positive controls for $CD11b_{PE}$ and $CD14_{FITC}$. Monocyte HLA-DR measurements were expressed as percentages of HLA-DR-positive monocytes and as means of fluorescence intensities (MFI) in relation to the entire monocyte population, thus reflecting the HLA-DR density per cell. Flow cytometry analysis was performed using Kaluza software V1.1 (Beckman Coulter, CA, USA). Setting gates were based on the internal negative population. The figure of analysis strategy for monocyte HLA-DR expression was presented in our previously published paper [13].

2.4. Cell Culture. 5×10^5 PBMCs were plated in two wells of a flat-bottomed 24-well plate (Nunclon, Aarhus, Denmark) in 1 mL of sterile RPMI 1640 tissue culture medium containing 5% heat-inactivated bovine serum, 1 mM of L-glutamine (Gibco, Grand Island, USA), and 1 mM sodium pyruvate. The cells in first well were not stimulated or treated. The cells in second well were stimulated with 1 pg/μL of LPS (Sigma, Missouri, USA). The plate was incubated at 37°C in 5% carbon dioxide for 24 h. Supernatants of the culture wells were sampled and stored at −80°C until use.

2.5. Measurement of Cytokine Levels. Cytokine levels of supernatants were measured with a human enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions. The ELISA kit of IL-10 was manufactured by Pierce Biotechnology, Illinois, USA. The IL-6, TGF-β1, and IL-17 were purchased from R&D Systems, Inc., Minnesota, USA. The IL-12, TNF-α, and IL-1β were purchased from Becton Dickinson, CA, USA. Cytokine responses were defined as the difference in supernatant levels with and without LPS stimulation. Negative responses were set as 0 pg/mL. Changes in cytokine responses were defined as the difference in cytokine response on day 7 minus the cytokine response on day 1.

2.6. Statistical Analysis. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software V17.0 for Windows (SPSS Inc., Illinois, USA). Differences in continuous variables between two groups were analyzed using the Mann-Whitney test, whereas differences

Table 1: Clinical characteristics of severe septic patients with and without low-dose steroid use (percentage, mean ± standard error mean).

	No-low-dose steroid ($n = 11$)	Low-dose steroid ($n = 18$)	p value
Age (years old)	72.3 ± 6.4	72.1 ± 2.2	0.132
Male	64	72	0.694
APACHE II score	24.5 ± 2.4	29.2 ± 1.5	0.142
History			
COPD	36	22	0.433
Heart failure	9	11	1.000
Hypertension	45	22	0.237
Diabetes mellitus	0	22	0.268
End stage renal disease	0	6	1.000
Liver cirrhosis	9	11	1.000
Infection source			0.595
Pneumonia	91	78	
Urinary tract infection	9	16	
Biliary tract infection	0	6	
Initial antibiotics for pathogens			0.367
Sensitive	36	61	
Resistant	36	28	
No pathogen isolated	27	11	

APACHE = acute physiology and chronic health evaluation; COPD = chronic obstructive pulmonary disease.

in categorical variables were analyzed using the chi-square test or Fisher's exact test. Differences in continuous variables in the same subjects were analyzed using the Wilcoxon signed-rank test. Generalized linear model analysis was used to determine the association between clinical characteristics and cytokine response. A p value < 0.05 was considered statistically significant.

3. Results

Of the final 29 enrolled subjects with severe sepsis, 18 patients were prescribed low-dose steroids and 11 were not (Table 1). In the low-dose steroid group, 12 patients survived for 28 days and six died (Table 2). All 11 patients that did not receive low-dose steroid therapy survived. The APACHE II score in low-dose steroid group was higher than no-steroid group, although the statistical analysis did not show difference. There were no significant differences in age, gender, histories, infection sources, and initial appropriateness of antibiotics between groups with and without low-dose steroid therapy. Patients administered low-dose steroid therapy displayed higher percentages of septic shock (94% versus 36%), compared with no low-dose steroid group. The rates of gastrointestinal bleeding, acute renal failure, thrombocytopenia, jaundice, bacteremia, and mortality were similar between the two groups.

Table 2: Adverse event and outcome (number, percentage).

	No-low-dose steroid ($n = 11$)	Low-dose steroid ($n = 18$)
Event		
Gastrointestinal bleeding	2 (18)	2 (11)
Acute renal failure	4 (36)	11 (61)
Shock	4 (36)	17 (94)*
Thrombocytopenia	3 (27)	5 (28)
Jaundice	0 (0)	1 (6)
Bacteremia	1 (9)	2 (11)
Mortality	0 (0)	6 (33)†

*$p = 0.001$ compared to no-low-dose steroid group, by Fisher's exact test.
†$p = 0.058$ compared to no-low-dose steroid group, by Fisher's exact test.

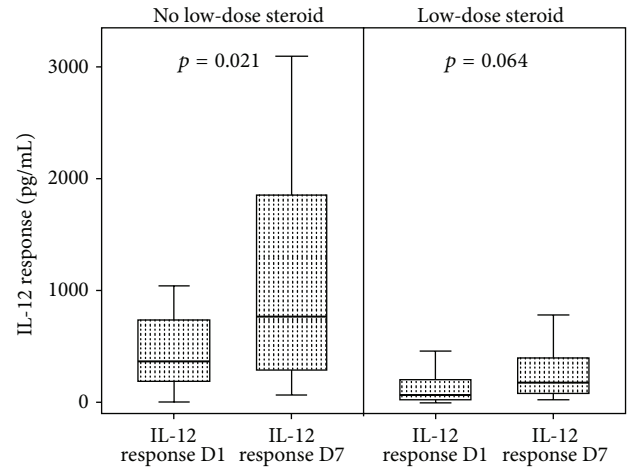

Figure 1: Interleukin- (IL-) 12 responses significantly recover in no low-dose steroid group. The boxplots show median of IL-12 response in day 1 and day 7 in groups with and without low-dose steroid treatment.

3.1. Cytokine Responses between Patients with and without Low-Dose Steroid Therapy. TNF-α response in the control group was significantly higher than in both patient groups (Table 3). However, IL-1β response in the control group was significantly lower than in both patient groups. IL-12 and TNF-α responses on days 1 and 7 in steroid group were significantly lower than in no-steroid group. There were no differences in IL-1β, IL-6, IL-10, IL-17, and TGF-β1 responses on days 1 and 7 between the two patient groups.

3.2. Change of Cytokine Responses from Day 7 to Day 1 in Subjects. IL-1β and TGF-β1 responses in patients administered low-dose steroid therapy decreased from day 7 to day 1 (Table 3). Compared to day 1, there were no changes in IL-6, IL-10, IL-12, IL-17, and TNF-α responses on day 7 in patients who received low-dose steroid therapy. IL-12 response in patients without low-dose steroid treatment significantly increased from day 7 to day 1 (Figure 1). Similarly, there were

Table 3: Cytokine responses (pg/mL, mean ± standard error mean) on days 1 and 7 in no-low-dose steroid, low-dose steroid, and control groups.

	No-low-dose steroid ($n = 11$)	Low-dose steroid ($n = 18$)	All patients ($n = 29$)	Controls ($n = 30$)
Day 1				
IL-1β	182.0 ± 18.6	200.0 ± 5.6	193.1 ± 7.8	110.8 ± 8.3†
IL-6	375.0 ± 40.2	416.2 ± 13.8	400.6 ± 17.5	385.4 ± 31.7
IL-10	340.4 ± 84.6	333.1 ± 74.1	335.8 ± 55.1	305.5 ± 29.4
IL-12	514.1 ± 151.8	141.3 ± 39.5*	282.7 ± 69.9	266.6 ± 50.5
IL-17	4.7 ± 4.3	2.6 ± 1.3	3.4 ± 1.8	1.0 ± 0.5
TGF-β1	26.3 ± 15.3	92.5 ± 28.7	67.4 ± 19.5	36.0 ± 9.9
TNF-α	404.9 ± 39.5	279.8 ± 41.2*	327.2 ± 31.4	540.8 ± 34.4†
Day 7				
IL-1β	189.8 ± 5.8	191.7 ± 5.7‡	191.9 ± 4.1	
IL-6	356.4 ± 35.2	410.3 ± 12.7	389.9 ± 15.9‡	
IL-10	339.3 ± 58.5	237.4 ± 41.0	276.0 ± 34.4	
IL-12	1145.7 ± 337.5‡	269.5 ± 57.7*	601.9 ± 152.1‡	
IL-17	0.8 ± 0.4	4.6 ± 1.9	3.2 ± 1.2	
TGF-β1	25.3 ± 16.2	28.2 ± 11.9‡	27.1 ± 9.4‡	
TNF-α	434.7 ± 14.3	367.1 ± 24.5*	392.8 ± 17.1	

IL = interleukin; TGF = transforming growth factor; and TNF = tumor necrosis factor.
$^{*}p < 0.05$ compared to no-low-dose steroid group, by Mann-Whitney test.
$^{\dagger}p < 0.05$ compared to all patients with severe sepsis, by Mann-Whitney test.
$^{\ddagger}p < 0.05$ compared within the same group on day 1, by Wilcoxon signed-rank test.

Table 4: Cytokine responses (pg/mL, mean ± standard error mean) on days 1 and 7 in survivors, nonsurvivors, and control groups.

	Survivors ($n = 23$)	Nonsurvivors ($n = 6$)	All patients ($n = 29$)	Controls ($n = 30$)
Day 1				
IL-1β	193.2 ± 9.2	192.9 ± 15.7	193.1 ± 7.8	110.8 ± 8.3*
IL-6	407.0 ± 21.4	376.2 ± 19.5	400.6 ± 17.5	385.4 ± 31.7
IL-10	298.1 ± 59.4	480.5 ± 132.0	335.8 ± 55.1	305.5 ± 29.4
IL-12	325.8 ± 84.1	117.6 ± 77.0	282.7 ± 69.9	266.6 ± 50.5
IL-17	3.5 ± 2.2	2.8 ± 2.3	3.4 ± 1.8	1.0 ± 0.5
TGF-β1	49.3 ± 17.8	136.8 ± 60.8	67.4 ± 19.5	36.0 ± 9.9
TNF-α	348.6 ± 35.4	245.1 ± 61.8	327.2 ± 31.4	540.8 ± 34.4*
Day 7				
IL-1β	192.0 ± 3.5	187.2 ± 15.6	191.9 ± 4.1	
IL-6	394.1 ± 19.3†	373.5 ± 21.8	389.9 ± 15.9†	
IL-10	273.5 ± 39.6	285.8 ± 74.7	276.0 ± 34.4	
IL-12	696.0 ± 185.7†	241.1 ± 108.2	601.9 ± 152.1†	
IL-17	2.7 ± 1.1	4.8 ± 4.6	3.2 ± 1.2	
TGF-β1	27.8 ± 10.7	24.5 ± 21.9	27.1 ± 9.4†	
TNF-α	403.9 ± 15.2	349.9 ± 59.5	392.8 ± 17.1	

IL = interleukin; TGF = transforming growth factor; and TNF = tumor necrosis factor.
$^{*}p < 0.05$ compared to all patients with severe sepsis, by Mann-Whitney test.
$^{\dagger}p < 0.05$ compared within the same group on day 1, by Wilcoxon signed-rank test.

no changes in IL-1β, IL-6, IL-10, IL-17, TGF-β1, and TNF-α production in patients treated without low-dose steroid therapy from day 7 to day 1.

In survivors with severe sepsis, IL-12 response increased and IL-6 response decreased significantly from day 7 to day 1. There were no changes in IL-1β, IL-10, IL-17, TGF-β1, and TNF-α production from day 7 to day 1 in survivors. All cytokine responses detected did not change from day 7 to day 1 in nonsurvivors (Table 4).

3.3. Association between the Change in Cytokine Responses and Low-Dose Steroid Therapy. Table 5 shows regression analysis results demonstrating the relationship between changes in IL-1β levels and low-dose steroid therapy and other clinical characteristics. APACHE II score levels were independently and positively associated with changes in IL-1β response ($B = 3.110$). Patients who were severely ill produced more IL-1β in PBMCs after 6 days compared with those who had milder illness. However, low-dose steroid therapy was not independently associated with the change in IL-1β levels. Low-dose steroid therapy did not influence IL-1β production in PBMCs of severe septic patients.

Low-dose steroid therapy was also independently and negatively linked to the increase in IL-12 after regression analysis ($B = -750.743$; Table 6). IL-12 recovery from day 7 to day 1 was lower in patients with low-dose steroid therapy. The presence of septic shock did not independently correlate with the change in IL-12 levels. Sex was independently related to the variation in IL-12 response. IL-12 recovery from day 7 to day 1 was high in male patients ($B = 447.838$).

Although TGF-β1 response in patients receiving low-dose steroidtherapy decreased from day 7 to day 1, the regression analysis did not find independent factors associated with the change in TGF-β1 levels (Table 7).

3.4. HLA-DR Expression with or without Low-Dose Steroid Therapy on Days 1 and 7. There was no difference in monocyte percentage, positive HLA-DR percentage in monocytes,

Table 5: Generalized linear model analysis for the change in IL-1β response to identify independent factors among clinical characteristics and steroid use.

	B	Change in IL-1β response or (rho)*	p value
Age	−0.689	0.068	0.154
Sex			0.775
Male	−4.358	−8.6, −94.5–94.1	
Female	0†	−13.1, −23.5–153.5	
APACHE II	3.110	0.292	0.006
Septic shock			0.083
Yes	−34.395	−10.8, −94.5–94.1	
No	0†	−3.8, −28.0–153.5	
Low-dose steroid			0.472
Yes	−13.113	−10.3, −94.5–94.1	
No	0†	2.2, −28.0–153.5	
Mortality			0.659
Yes	7.850	−8.5, −94.5–94.1	
No	0†	−9.9, −38.4–153.5	

IL = interleukin; APACHE = Acute Physiology and Chronic Health Evaluation.
*Data are presented as the median and range (in pg/mL) for categorical variables or the Spearman rank correlation coefficient (rho) value for continuous variables.
†0 is set as a reference.

Table 6: Generalized linear model analysis for the change in IL-12 response to identify independent factors among clinical characteristics and steroid use.

	B	Change in IL-12 response or (rho)*	p value
Age	−2.369	0.015	0.727
Sex			0.036
Male	447.838	253.725, −432.0–2059.9	
Female	0†	58.3, −813.8–1023.4	
APACHE II	−5.300	−0.316	0.095
Septic shock			0.083
Yes	482.418	176.4, −432.0–2059.9	
No	0†	241.6, −813.8–1928.9	
Low-dose steroid			0.003
Yes	−750.743	87.6, −432.0–679.7	
No	0†	352.3, −813.8–2059.9	
Mortality			0.574
Yes	−140.572	49.3, −432.0–679.7	
No	0†	224.6, −813.8–2059.9	

IL = interleukin; APACHE = Acute Physiology and Chronic Health Evaluation.
*Data are presented as the median and range (in pg/mL) for categorical variables or the Spearman rank correlation coefficient (rho) value for continuous variables.
†0 is set as a reference.

and MFI of HLA-DR between patients treated with or without low-dose steroids on day 1 or 7 (Figure 2). In addition, there was no change in monocyte percentage, positive HLA-DR percentage in monocytes, and MFI of HLA-DR in patients treated with or without low-dose steroids from day 7 to day 1.

4. Discussion

Previous studies often used *in vitro* cultures to test the effectiveness of different doses of steroids [4] or repeatedly measured plasma levels between the patients receiving or not receiving steroids at specific times [5]. These studies have a common fault; they indirectly detect the function or response of immune cells after a course of low-dose steroid therapy. Since circulating cytokines can be produced by immune and nonimmune cells, such as smooth muscle cells and endothelial cells [17], circulating cytokine levels only reflect a relatively broad response.

In this study, we demonstrated that a complete course therapy with low-dose steroid was associated with decreased IL-12 production in PBMCs from patients with severe sepsis. In contrast to our results, a 6-day crossover study with serial detecting plasma cytokine levels in patients with septic shock reported that the inhibitory effect of hydrocortisone infusion did not decrease plasma IL-12 level [18]. The first possible reason for this result is that only 3 days of hydrocortisone infusion was administered to each group; therefore, the effect of low-dose steroid did not have a chance to develop. Second, Ficoll density gradient centrifugation for monocytes isolation resulted in lower cell function than by positive selection by magnetic microbeads [19]. The function of IL-12 production from PBMCs in this study might be influenced by Ficoll solution. Other reasons may be that there is increased IL-12 production in dendritic or human B-lymphoblastoid cells, resulting in similar amounts of circulating IL-12.

In this study, we did not find an association between IL-6 response and low-dose steroid therapy. This was similar to a study by Oppert et al. They found that IL-6 production in LPS-stimulated diluted whole blood transiently fell from day 1 to 3 and returned after day 5 in a low-dose hydrocortisone therapy group [5]. In terms of circulating cytokine levels, there were no significant differences in plasma IL-6 levels between treatment groups of continuous 6 h infusion of endotoxin + hydrocortisone or endotoxin + saline in an animal study [20]. Furthermore, a double-blind, randomized, placebo-controlled study showed similar results [21]. Two doses of hydrocortisone (100 mg per 8 h) were administered after bilateral total knee replacement in the study group. IL-6 levels were 40% lower in the study group after 10 h but returned to levels similar to that of the control group at 24 h. However, other studies have reported different results. Stress dose of hydrocortisone infusion in patients with septic shock significantly decreased plasma IL-6 levels on day 5 between the steroid and placebo groups [22]. Hydrocortisone infusion reduced plasma IL-6 levels in a 6-day crossover

Table 7: Generalized linear model analysis for the change in TGF-β1 response to identify independent factors among clinical characteristics and steroid use.

	B	Change in TGF-β1 response or (rho)*	p value
Age	0.296	0.039	0.789
Sex			0.351
Male	−32.608	−13.4, −253.9–173.7	
Female	0†	0.0, −133.6–0.0	
APACHE II	−5.110	−0.282	0.051
Septic shock			0.350
Yes	42.598	0.0, −253.9–173.7	
No	0†	0.0, −155.8–66.8	
Low-dose steroid			0.388
Yes	−36.088	−13.4, −253.9–66.8	
No	0†	0.0, −155.8–173.7	
Mortality			0.066
Yes	−75.079	−96.5, −253.8–13.4	
No	0†	0.0, −229.0–173.7	

TGF = transforming growth factor; APACHE = Acute Physiology and Chronic Health Evaluation.
*Data are presented as the median and range (in pg/mL) for categorical variables or the Spearman rank correlation coefficient (rho) value for continuous variables.
†0 is set as a reference.

study in patients with septic shock [18]. Generally, low-dose steroid therapy may influence plasma IL-6 levels but not IL-6 response in PBMCs, based on current evidence.

The effect of steroid therapy on IL-10 production is controversial. Stimulated monocyte production of IL-10 was enhanced at low concentrations with glucocorticosteroid therapy [23]. This effect was not observed in our study. IL-10 production in stimulated PBMCs did not differ between patients receiving or not receiving low-dose steroid therapy on day 1 or 7. Even after 6 days of therapy, IL-10 response did not change in the low-dose steroid group. Moreover, dexamethasone inhibited LPS-stimulated release of IL-10 from diluted whole blood of septic patients in a dose-dependent manner [4]. After *in vivo* administration of high or low amounts of cortisol, high cortisol therapy further increased LPS-induced IL-10 expression in isolated monocytes from healthy participants, whereas low cortisol therapy decreased Il-10 expression [24]. More studies are needed to determine the role of low-dose steroid therapy on IL-10 production in patients with severe sepsis.

Endogenous cortisol is one of the main anti-inflammatory mediators induced by our central nervous system during severe sepsis. It has been described that steroids have downregulating effects on monocyte HLA-DR expression [25–27]. High endogenous cortisol levels observed in septic shock patients may play a role in the loss of monocyte HLA-DR expression via its effect on HLA-DR transcription. However, monocyte HLA-DR expression seems not to be influenced by low-dose steroid therapy. In this study, there was no change in monocyte percentage, positive HLA-DR percentage in monocytes, and MFI of HLA-DR in patients with low-dose steroid therapy from day 1 to 7. Similar results have been reported by Keh et al. [18]. Patients with low-dose hydrocortisone therapy showed unchanged monocyte HLA-DR expression. More studies are needed to confirm our and Keh et al.'s results.

Cortisol can influence the immune system and is crucial for the host for defense against pathogens. The endocrine system may be also influenced by the immune system. During inflammation, cytokines mediate a high glucocorticosteroid output with regulation from the neuroendocrine to the immune-endocrine system [28]. As a result, high levels of adrenal glucocorticosteroid are vital in preventing an uncontrolled inflammatory response to cytokines.

The IL-12 response from PBMCs was restored in patients who survived severe sepsis [13]. Severe septic survivors produced more IL-12 from LPS-stimulated PBMCs than nonsurvivors [29]. The main immunological function of IL-12 is to enhance native T lymphocyte differentiation to type 1 help T (Th1) cells. Th1 cells secrete interferon-γ that regulates macrophage and natural killer (NK) cell activation, stimulates immunoglobulin secretion by B cells, and enhances Th1 cell differentiation. In this work, low-dose steroid therapy resulted in decreased IL-12 production. This might explain why low-dose therapy was associated with an increase in adjusted hospital mortality in SSC database [10]. Decreased IL-12 response in patients receiving low-dose steroid therapy may depress a protective effect by decreased cellular immunity and phagocytic functions.

There were two limitations in this work. First, the percentage of septic shock was different between steroid and no-steroid groups. The reason why steroid group had higher percentage of shock is that patients with septic shock are appropriate for steroid treatment according to guidelines. In septic shock group, not in no-shock group, patients with low-dose steroid had significantly lower change of IL-12 response than those without low-dose steroid. Although generalized linear model analysis was used to exclude the confounding effect of shock, there was not a directly statistical analysis to demonstrate low-dose steroid affected IL-12 response recovery in patients without septic shock. Second, patients with low-dose steroid treatment have received steroid when their blood was drawn on day 1. Although the time from onset of steroid treatment to blood sampling was short and not more than 48 hr, cytokine responses and HLA-DR expression might be influenced.

5. Conclusions

IL-12 response observed in PBMCs increased from day 7 to day 1 in severe septic patients. We demonstrated that a course of low-dose steroid therapy influenced IL-12 production from *in vitro* LPS-stimulated PBMCs of severe septic patients. Low-dose steroid therapy was associated with less increase of IL-12 response. There was no correlation between low-dose steroid therapy and monocyte HLA-DR expression.

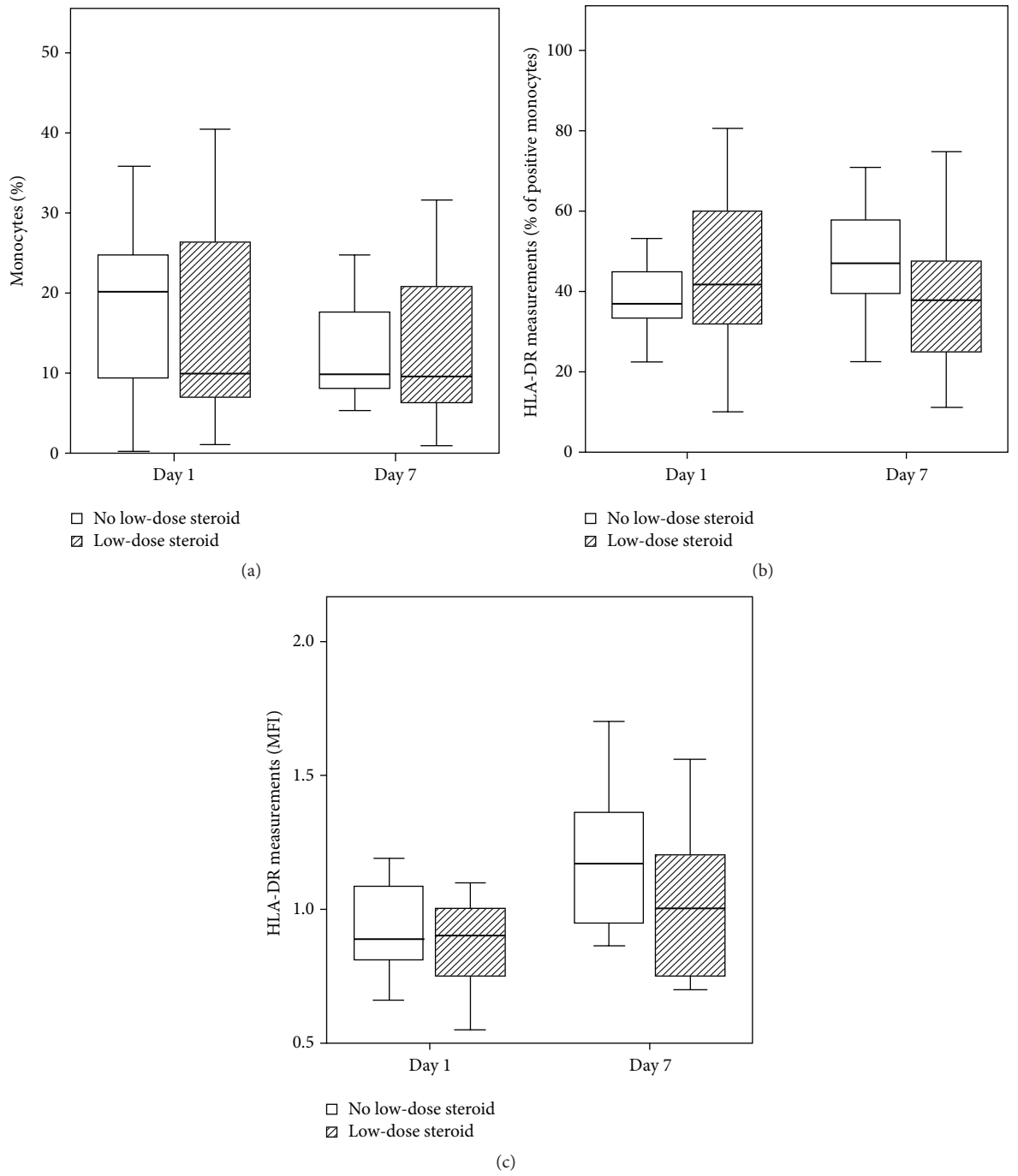

Figure 2: No change of HLA-DR expression between groups of no low-dose steroid and low-dose steroid. Boxplots of (a) monocyte percentage, (b) positive HLA-DR percentage in monocytes, and (c) MFI of HLA-DR are shown in patients treated with or without low-dose steroid therapy on days 1 and 7. HLA= human leukocyte antigen; MFI= means of fluorescence intensities.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Huang-Pin Wu designed this study and wrote this paper. Chi-Chung Shih and Duen-Yau Chuang participated in data analysis, interpretation of results, and discussion. Tien-Hsing Chen participated in discussion.

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Review Article

Neuroimmunomodulation in the Gut: Focus on Inflammatory Bowel Disease

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Intestinal immunity is finely regulated by several concomitant and overlapping mechanisms, in order to efficiently sense external stimuli and mount an adequate response of either tolerance or defense. In this context, a complex interplay between immune and nonimmune cells is responsible for the maintenance of normal homeostasis. However, in certain conditions, the disruption of such an intricate network may result in intestinal inflammation, including inflammatory bowel disease (IBD). IBD is believed to result from a combination of genetic and environmental factors acting in concert with an inappropriate immune response, which in turn interacts with nonimmune cells, including nervous system components. Currently, evidence shows that the interaction between the immune and the nervous system is bidirectional and plays a critical role in the regulation of intestinal inflammation. Recently, the maintenance of intestinal homeostasis has been shown to be under the reciprocal control of the microbiota by immune mechanisms, whereas intestinal microorganisms can modulate mucosal immunity. Therefore, in addition to presenting the mechanisms underlying the interaction between immune and nervous systems in the gut, here we discuss the role of the microbiota also in the regulation of neuroimmune crosstalk involved in intestinal homeostasis and inflammation, with potential implications to IBD pathogenesis.

1. Introduction

The enteric nervous system (ENS) constitutes a major autonomic division of the nervous system that provides the intrinsic innervation of the gut, capable of controlling different functions, such as motility, mucosal secretion and absorption, mucosal growth, local blood flow, and the immune function [1]. The ENS can be influenced by the central nervous system (CNS), establishing a two-way relationship treaded by the brain-gut axis. Actually, all basic gastrointestinal (GI) functions can be regulated by the ENS, but the coordination of the gut function and the maintenance of the homeostasis of the organism both require a communication between the GI tract and the CNS [2]. The ENS consists of two interconnected networks of ganglia and fibers encircling the GI tract, arranged in a peculiar way, which confers the ability to mediate its own reflexes. In this context, evidence shows that ENS can work independently of the CNS [3]. Because of these properties, ENS is a primary regulator of GI functions and has been referred to as a second brain in humans [3]. However, despite the ability of the ENS to regulate motility and secretion in an autonomous way, there are many connective links with the CNS, working in a bidirectional fashion [4].

Nerve cells located within the mucosa are in close proximity to immune cells, where they produce and respond to several common mediators [5, 6]. Upon ENS activation, mucosal immune cells expressing receptors for neurotransmitters can be stimulated to migrate, degranulate, differentiate, or secrete immunoglobulins, for example, [7–9]. Therefore, the communication between the ENS and the immune system within the mucosa participates in the

control of major GI functions but can also be associated with pathological conditions, such as inflammatory bowel disease (IBD).

2. Enteric Nervous System

The ENS, commonly called "the little brain of the gut," is a constituent of the peripheral nervous system which is composed by an intrinsic network containing enteric neurons cell bodies, intestinal cells of Cajal (ICC), interneurons and motor neurons, and enteric glial cells (EGC). The latter have been extensively studied in recent years and consist of small cells with stellate shape that is associated with neuron cell bodies and nerve fibers in intraganglionic connections. Evidence supports that EGC are very similar to astrocytes, not only morphologically, but also functionally [10]. All these elements are grouped into ganglia and interconnected by bundles of nerve processes forming plexuses, including the myenteric (Auerbach's) and submucosal (Meissner's) plexus.

The myenteric plexus extends from the upper esophageal to the external anal sphincter, situated between the longitudinal and the circular muscle layer. The submucosal plexus is restricted to the submucosa of the small and large intestines. In human, for instance, two ganglionated submucosal plexuses can be distinguished. Thereby, there is an internal submucosal plexus, which lies in the inner half of the submucosa, and also an external submucosal plexus, this one situated close to the inner border of the circular muscle layer [11] (Figure 1).

In the present review, the current knowledge and the clinical implication of ENS in IBD will be discussed.

3. Mediators of the Enteric Neurons System

Neurotransmitters are molecules produced by neurons that play a role in the transmission of information cell to cell, in maintaining stimulus of impulses, and act in the neuromuscular junction. When an action potential reaches the terminal button of a presynaptic neuron, a voltage-dependent calcium channel opens, resulting in the release of neurotransmitters in the synaptic cleft [12, 13]. These molecules, also known as neuropeptides, including acetylcholine, serotonin, substance P, corticotropin-releasing hormone, and vasoactive intestinal peptide (VIP), are distributed throughout the gut and participate in normal homeostasis as well as in inflammatory processes [14].

Acetylcholine (ACh) plays a role in both CNS and in ENS upon ligation with ACh receptors, ligand-dependent cation channels, of which the two major classes are the muscarinic and the nicotinic receptors [15, 16]. The activation of ACh receptors by binding to ACh determines the depolarization of the postsynaptic neuron and the initiation of a new action potential [17, 18].

Another neurotransmitter, abundant in intestinal neuroendocrine cells, is serotonin (5-hydroxytryptamine, 5-HT). About 95% of 5-HT in the human body is found in GI tract and its action influences luminal contents and secretions [19]. The most frequent component of the enteroendocrine cell population is the enterochromaffin cell (EC), estimated to contain 90% of the total intestinal 5-HT, while 10% is in enteric neurons [19–21]. Once released basolaterally, 5-HT can perform multiple functions, including action on primary intrinsic afferent neurons initiating peristaltic reflex, the stimulation of cholinergic neurons to release acetylcholine, resulting in smooth muscle contraction, and the stimulation of inhibitory nitrergic neurons to release NO, which results in smooth muscle relaxation [22]. In addition, 5-HT participates in potential mucosal protecting processes, stimulating active ion, mucus, and fluid secretion. The secretory effect of 5-HT is mediated by epithelial 5-HT2 receptors and neuronal 5-HT1P, 5-HT3, and 5-HT4 receptors [22]. In the GI tract, the abnormal secretion of 5-HT has been associated with various effects, such as nausea, vomiting, and alterations in the intestinal secretion and peristalsis [19], indicating that this neuroendocrine transmitter plays an important role in the regulation of gastrointestinal functions. Interestingly, the major source of 5-HT, EC cells, also expresses toll-like receptors, which make them capable of sensing microorganisms [23, 24].

Substance P (SP) is localized in enteric nerves distributed throughout the gut and present in myenteric and submucosal plexuses [25, 26]. The effects attributed to SP, such as regulation of mucosal permeability [27], motility [28], secretion [29], epithelial cell proliferation [30], and inflammation [31], are initiated upon ligation with G-protein-coupled NK-1R, which is present in both the small and large bowel of animals and humans [32, 33]. In regard to intestinal inflammation, SP-NK-1R-induced proinflammatory signaling was shown to result in the production of a downstream cascade of proinflammatory molecules mediated by the activation of NF-kappa B [34, 35], or p38 mitogen-activated protein kinase [36]. It is interesting to note that NF-kappa B can also modulate NK-1R expression [37]. SP has been identified also in immune cells, such as dendritic cells, mononuclear phagocytes, and lymphocytes [28, 38–41], while NK-1R can be present in T and B cells, macrophages, dendritic cells, neutrophils, natural killer cells, and eosinophils [28, 39, 42–45]. Moreover, SP and NK-1R can promote inflammation by regulating intestinal angiogenesis through the increase in the expression of CCN1 (CYR61) [46], which is upregulated in the colon from UC patients [47].

Nitric oxide (NO) is regarded as a cellular signaling molecule, which can play different roles in the GI tract, such as participating in the maintenance of mucosal integrity and also regulating vascular tone and the mucosal blood flow [48]. NO is catalyzed by one of the isoforms of nitric oxide synthase (NOS), of which the GI tract expresses constitutively two: endothelial NOS (eNOS) and neuronal NOS (nNOS) [49]. An additional isoform is the inducible isoform (iNOS), which is upregulated in response to inflammation and other stimuli. The increase in NO concentration, in turn, results in the production of reactive oxygen species and consequently also potential oxidative stress [50]. In fact, increases in NO concentration have been associated with harmful effects in the GI tract, including human IBD and also experimental colitis [51]. Interestingly, the increase in both iNOS expression and NO production in patients with ulcerative colitis was shown

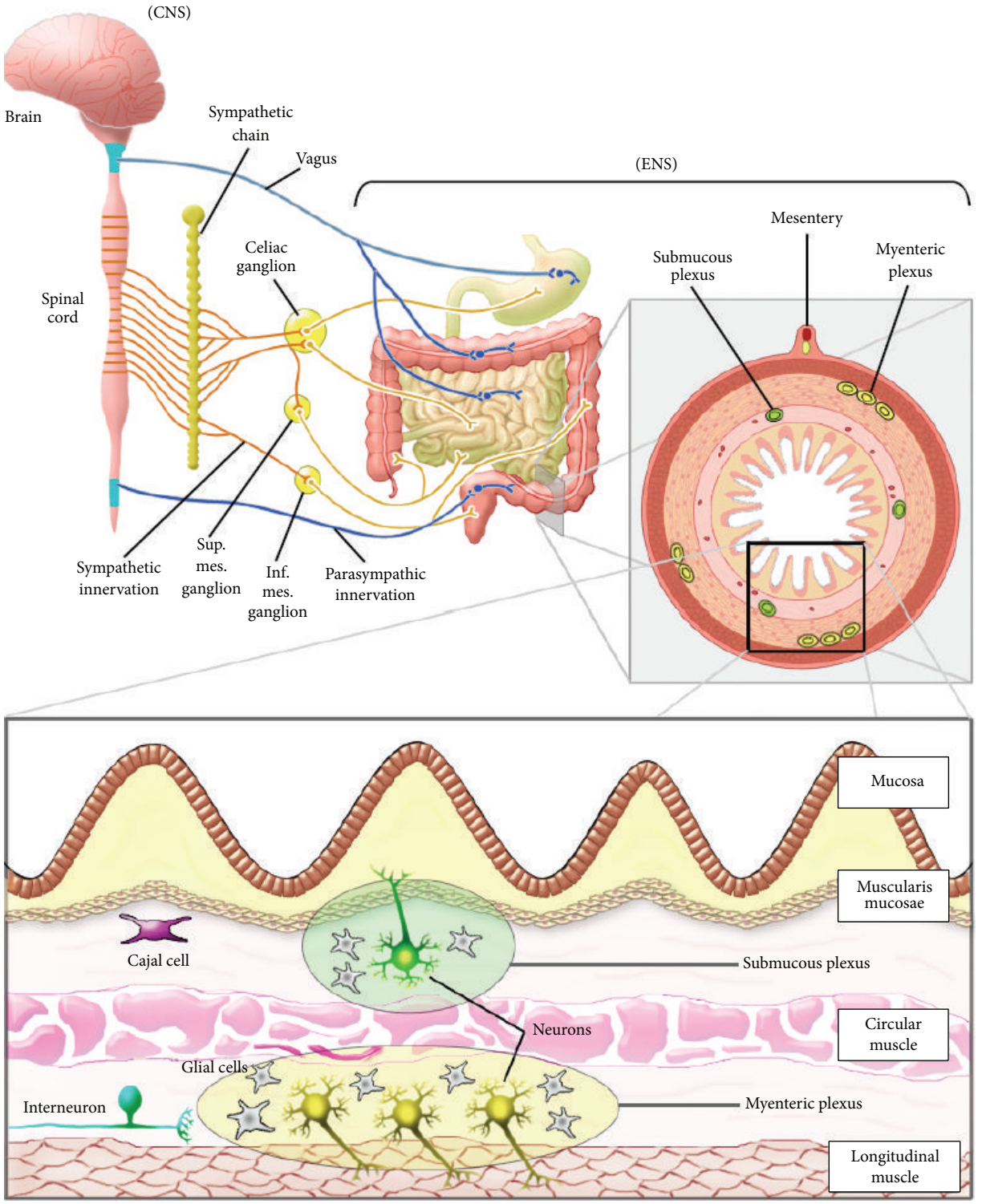

Figure 1: Schematic illustration showing the interaction between the central nervous system (CNS) and the enteric nervous system (ENS). The sympathetic and parasympathetic innervations interact with neurons in the gastrointestinal tract, passing the sympathetic innervations first through the celiac ganglion and the superior mesenteric ganglion (sup. mes. ganglion) and the inferior mesenteric ganglion (inf. mes. ganglion). The quadrant below the CNS and the ENS represents the intestinal mucosa and its myenteric and submucosal plexuses.

to be secondary to enteroglial-derived S100B protein upregulation. This information unveils an unexpected mechanism by which the enteric glia mediates mucosal NO-dependent inflammatory responses [52]. The role of NO in intestinal homeostasis and inflammation is further reinforced by the fact that VIP is released from nerve terminals containing NOS. Such peptides are thought to comprise a nonadrenergic and noncholinergic nerve transmission circuit within the gut [53, 54].

4. Enteric Nervous System in the Pathogenesis of Inflammatory Bowel Disease

During the course of IBD, the marked immune cell infiltration and the activation of mechanisms that modulate cell turnover within the intestinal epithelium can lead to permeability changes with potential disruption of the intestinal barrier [55–57]. This milieu of changes, which in certain levels contributes to the pathogenesis of IBD, can further progress and ultimately affect also the morphology and function of the ENS [58]. Abnormalities such as axonal rupture of nerve fibers, damage of neuronal cell bodies, hyperplasia of EGC, and increase of axonal necrosis of gut nerves have been associated with IBD [59–62]. Villanacci et al., for example, observed differences in the number of neuronal cell bodies, ICC and EGC in patients with Crohn's disease (CD) and ulcerative colitis (UC). Interestingly, abnormalities such as a reduction of enteroglia, found in noninflamed areas of the intestine, led to the hypothesis of a pathogenic role of the ENS in IBD [63]. Ohlsson et al. corroborated these findings and further demonstrated the presence of visceral ganglioneuritis, in addition to atrophy and vacuolar degeneration of ICCs in the small bowel of patients with CD [64]. On the other hand, in another study investigating the noninflamed tissue of CD patients, the transmitter colocalization patterns in rectal submucosal neurons by immunohistochemistry revealed an increase in the vasoactive intestinal polypeptide (VIP) population, extensive colocalization of choline acetyltransferase (ChAT) and NOS, and hypertrophied calcitonin gene-related peptide (CGRP) fibers [65], supporting the occurrence of adaptive alterations in the ENS in CD.

In the context of experimental colitis, enteric nervous abnormalities have also been reported in animal models. For example, results from a study using trinitrobenzene sulfonic acid- (TNBS-) induced colitis have shown that, in the beginning of inflammation, 20% of myenteric neurons are rapidly lost [66], and neuronal loss has been attributed to cell death induced by the inflammatory process consequent to TNBS-induced colitis and associated with infiltration of neutrophils [66, 67]. Hence, it is probable that the activation of immune cell-related molecules involved in IBD pathogenesis also might be responsible for the abnormalities of ENS.

5. Immune Cells Interaction with ENS

The presence of neuropeptides and neurotransmitter receptors on immune cells represents a strong indication of the integration between the ENS and the immune system.

5.1. T Cells. In normal conditions, mucosal T cells respond to different environmental challenges orchestrating the immune response in an adaptable fashion [68]. In IBD, such plasticity of T cells appears to be compromised, resulting in chronic inflammation [69]. Currently, in CD, the immune response has been regarded as a mixture of a T helper type 1 (Th1) and Th1/Th17 phenotypes [70], while in UC it comprises an atypical Th2 phenotype, with the addition of Th9 [71], and a less prominent Th17 response [72]. Recently, complex modulatory mechanisms reciprocally involving the ENS and the mucosal immune system have been recognized [73]. For instance, the vagus nerve appears to play an important role in this integrative process [74, 75] and, when stimulated, it acts as an anti-inflammatory promoter activating sympathetic neurons in the mesenteric ganglion that release noradrenalin, which activates T cells. These T cells, defined as memory cells, in turn, release acetylcholine (Ach) that inhibits proinflammatory cytokines from macrophages [76]. Particularly, in physiological conditions, T cells have been seldom seen in the proximity to ENS. Nevertheless, Sayani et al. demonstrated that, in the context of experimental intestinal inflammation, mucosal T cells increased, but being consistently excluded from ganglia. Such effect has been attributed to the expression of Fas-ligand (Fas-L) on enteric neurons, which appears to protect them against Fas-Fas-L-induced apoptosis, possibly further contributing to the resolution of inflammation [77]. Taken together, these evidences support the idea of a neural information system capable of controlling innate and adaptive immune responses.

Another interesting example of this integration is the evidence of hypothalamic-pituitary-adrenal axis regulation of intestinal inflammation [78], through the anti-inflammatory effects of glucocorticoids [79]. In IBD, the activated inflammatory cascade has been shown to affect GI motility and function [80], providing another indication of an intimate communication between ENS and the mucosal immune system (Figure 2).

5.2. Macrophages. In the intestinal mucosa, resident macrophages are usually present in the lamina propria where they preferentially locate in the subepithelial area to constitute the first line of defense against potentially harmful external stimuli [81]. Nevertheless, macrophage subsets are distributed also below the epithelial layer, towards the submucosa and muscularis externa, exhibiting distinct phenotypes and probably specific functions [82].

In the gut, vagus nerve stimulation has anti-inflammatory properties, also known as cholinergic anti-inflammatory pathway, with influence on diverse immune-mediated disorders [83]. For example, vagus nerve activation by electrical stimulation and systemic nicotinic receptor agonists was shown to abate intestinal inflammation, by reducing the production of proinflammatory cytokines by macrophages [84]. Recently, the interaction between vagal efferents and intestinal macrophages has been investigated, but no clear evidence of direct modulation could be demonstrated. Hence, researchers proposed that vagal modulation of intestinal

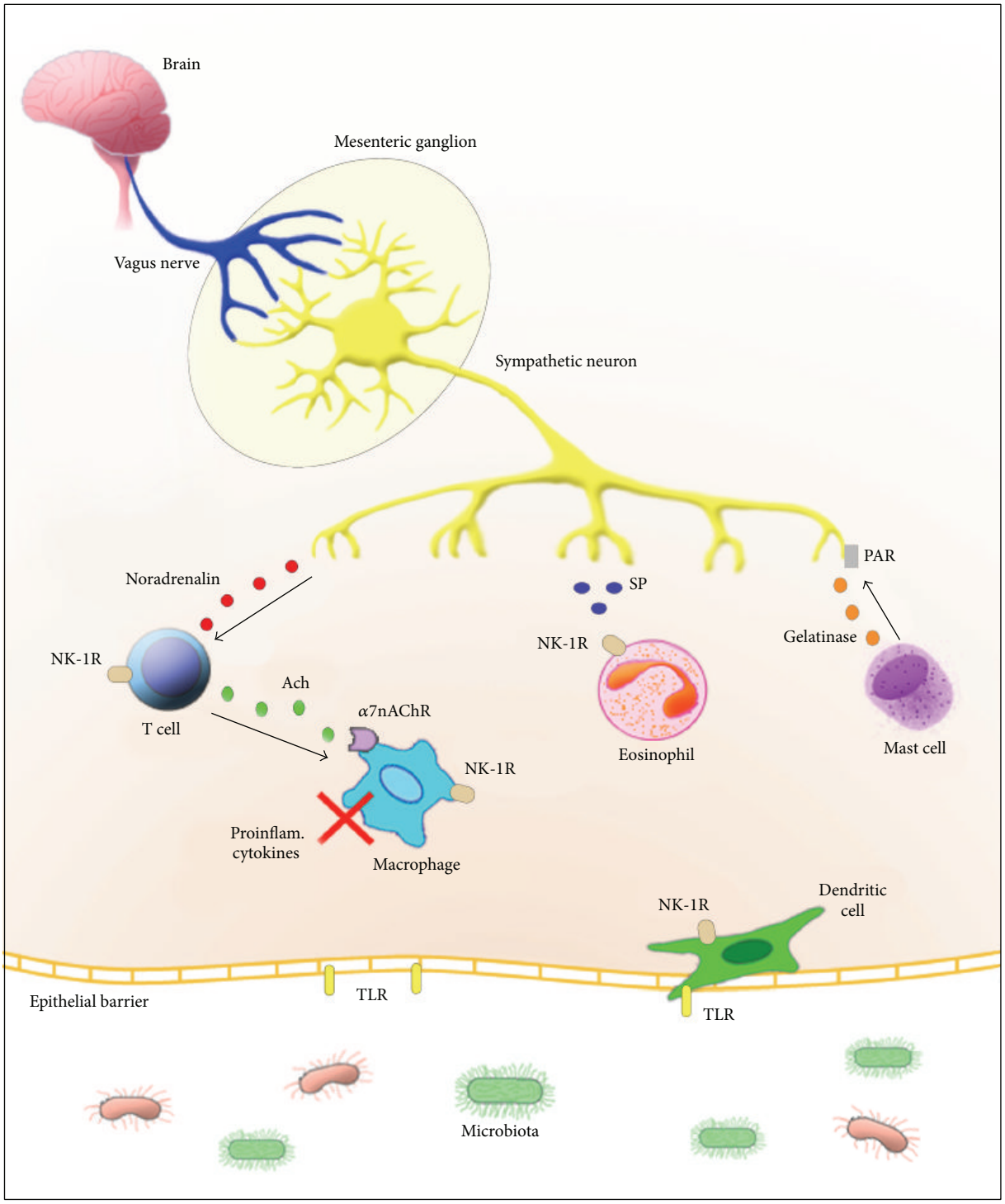

FIGURE 2: Interaction between the enteric neuron system (ENS) and mucosal immune cells. Upon vagus nerve stimulation, the sympathetic innervation secretes neurotransmitters that can modulate immune cells and the inflammatory response. The intestinal microbiota also participates in the inflammatory response fine-tuning the interaction between the ENS and mucosal immune cells.

macrophages could actually be indirect, probably via cholinergic and nitrergic/VIPergic enteric neurons [85].

TNF-alpha, a prototypical Th-1-type of proinflammatory cytokine, associated with IBD and particularly with CD [86], can be modulated by the vagus nerve, through the inhibition of macrophage release. Notably, Wang et al. reported that the nicotinic acetylcholine receptor alpha 7 subunit (α7nAChR) is essential in acetylcholine inhibition of TNF-alpha production by macrophages [87], revealing a mechanism of neuromodulation of the immune response. In another study

on the subject, the anti-inflammatory action of the vagus nerve in the intestine was shown to be dependent on its interaction with cholinergic myenteric neurons in intimate association with the muscularis macrophages. In addition, it has been suggested that resident macrophages expressing α7nAChR would probably be the ultimate intestinal target of such anti-inflammatory pathway [88].

5.3. Neutrophils. Like T cells, neutrophils are rarely observed in submucosal and myenteric plexuses in normal conditions, but during chronic active IBD these inflammatory cells accumulate and infiltrate the mucosa, contributing to the tissue injury [89] and possibly also affecting the ENS.

In experimental colitis in rats, induced by dinitrobenzene sulfonic acid (DNBS) administration, Sanovic et al. demonstrated a significant neuronal reduction in the inflamed segments in the first 24 hours, with less than half of neurons remaining by days 4 to 6 and thereafter, when inflammation had diminished. The neuronal damage was associated with the early accumulation of neutrophils and eosinophils within the ganglia, an effect more prominent in the submucosal ganglia [90]. In another experimental study with DNBS-induced colitis, mice treated with anti-neutrophil antibody had a significant attenuation of tissue damage and a greater number of neurons compared to nontreated colitic mice [67]. In conjunction, these data suggest that neutrophils might participate in the loss of ENS neurons during inflammatory intestinal conditions, including IBD.

5.4. Eosinophils. In the past three decades, relevant evidences point to an important role of eosinophils in IBD [91–94]. Smyth et al., studying different clinical stages of IBD, demonstrated that the major basic protein (MBP, a cationic protein released by eosinophils, which can be cytotoxic in high concentrations), as well as eosinophils, localizes to nerves and ganglia in the mucosa of patients with refractory disease [95]. Moreover, an increased expression of eotaxin-3 and ICAM-1, molecules involved in tissue eosinophilia and leukocytes transmigration, respectively, was detected in the same mucosal location, in the vicinity of nerves and ganglia [96]. In refractory CD patients, eosinophils have been found close to nerves within the smooth muscle layer. Furthermore, eosinophils localized specifically to SP and ChAT nerves, in CD mucosa, suggesting an indirect role for eosinophils also as mediators of smooth muscle contraction and gut motility [95]. Eosinophils have also been implicated in the pathophysiology of UC, and the involvement in mucosal inflammation and destruction was suggested to be associated with SP innervation and the neurokinin-1 receptor (NK-1R) expression, most marked in areas of mucosal accumulation of eosinophils [97, 98].

5.5. Mast Cells. Mast cells play an important role in innate and adaptive immune responses by regulating the allergic reaction and defense against pathogens. Their growth and proliferation are regulated by cKit ligand stem cell factor (SCF), nerve growth factor (NGF), IL-3, IL-4, IL-9, and IL-10 [99]. Under physiological conditions, mast cells are present in the mucosa, submucosa, and the circular muscle layer [100] and play a role in allergic diseases by releasing proteases, cytokines, and chemokines [101].

Increased mast cell numbers are observed in the proximity of mucosal enteric nerve fibers in a model of visceral hypersensitivity in rats [102]. In a model of food allergy in mice, Lee et al. observed that nerve fibers expressing the neurotransmitter CGRP (calcitonin gene-related peptide) were increased and colocalized to mucosal mast cells in the colonic mucosa [103]. The proximity of mast cells to mucosal enteric nerve fibers has also been described in adult patients with irritable bowel syndrome (IBS) [104], a disease in which clinical manifestations notably overlap with IBD [105]. In an animal model of IBS, for instance, Barbara et al. showed that mucosal mast cells are capable of exciting nociceptive visceral sensory nerves, suggesting their implication in visceral hypersensitivity in IBS [106]. Ileal segments of patients with CD were shown to host a marked number of mast cells displaying piecemeal degranulation associated with ICC, in the muscularis. Of note, various types of injury were described in ICC, probably due to direct contact with mast cells and the chronic release of their potentially cytotoxic granule contents [107].

6. Purinergic Receptors

Purinergic receptors, also known as purinoceptors, are transmembrane receptors including the P1 and P2 subtypes. P2 is composed of two subforms, namely, P2Y and P2X. P2Y, and its variants, is a G-protein coupled receptor, while P2X, and its variants, is a ligand gate ion channel [108]. P2X7 is the most studied purinoceptor, and it has been implicated in the induction of caspase activity, cytokine secretion, and cell death. The ligand for P2X7 is adenosine triphosphate (ATP), a molecule that at high concentrations functions as a danger signal associated with tissue inflammation and damage [108], therefore constituting a damage-associated molecular pattern (DAMP). Gulbransen et al. studied the activation of enteric neuronal P2X7 receptor during inflammation in animal models and demonstrated that the myenteric neuronal density decreased during colitis, but with a pretreatment with oxidized ATP (an antagonist of P2X7 receptor) there was a protection against inflammation-induced neurons loss. On the other hand, using BzATP, an agonist of P2X7 receptor, neural packing density was reduced [109]. This phenomenon was also observed when ChAT-, calbindin-, calretinin-, anti-HuC/D-, and NOS-neurons, in which cells express P2X7 receptor, were decreased during colitis [110]. Gulbransen et al. also demonstrated that, in addition to the P2X7 receptor expression in myenteric neurons, these cells also express Panx-1, absent in EGC [109]. The P2X7 receptor expression has been associated with cell death during intestinal inflammation in human and experimental IBD [111], and the activation of P2X7 receptor-Panx-1 was proposed to contribute to neuron death by activation of a complex of caspases. In this regard, the release of ATP by Panx-1 also mediated death of EGC by phospholipase-C (PLC) pathway initiated by P2Y1 [109]. Furthermore, it

has been shown that the rapid loss of myenteric neurons involves not only caspase-dependent pathway but also other multiprotein complexes, such as the inflammasome [109]. In accordance with this, blocking either P2X7 receptor or Panx-1, associated with inflammasome triggering, was shown to prevent neurons death [109]. Other purinergic receptors have been described within enteric neurons. In the myenteric and submucosal plexuses, the ChAT, calbindin, calretinin, and NOS neurons also express P2X2 receptor, which can bind to ATP and mediate synaptic transmission [112]. In the myenteric plexus, the P2X6 receptor is expressed in neurons that resemble Dogiel type II neurons [113]. The G-protein coupled purinergic receptor P2Y2 is distributed in both plexuses, in neurons and fibers. In the myenteric plexus of small intestine of guinea pig, P2Y2 receptor is associated with neuropeptide-Y and calretinin [114]. In a model of chronic inflammatory pain, this purinergic receptor is increased in peripheral cutaneous sensory neurons that innervate injured tissue [115]. Recently, it has been demonstrated that ATP liberated from the gut epithelium during cell stressful stimuli, can mediate excitation of visceral afferents through P2X receptors [116], and also stimulate mouse and human visceral nociceptors through P2Y receptors [117]. These findings appear to implicate ATP in the generation of functional GI alterations, as a neurogenic component of the inflammatory process. In addition, ATP, regarded as DAMP, triggers mechanisms downstream of P2X7 and mediates the inflammasome activation, probably contributing to the maintenance and amplification of the inflammatory response [118, 119].

7. Enteric Glial Cells

Under physiologic conditions, intestinal barrier is relatively impermeable, but during pathologic conditions, barrier disruption has been associated with the development of GI diseases, including inflammatory disorders [120]. Regarding the intestinal barrier, results of recent studies suggest an important contribution of EGC in the maintenance of normal functions. EGC are abundant in GI tract [121, 122] and are in close proximity to the intestinal epithelial border and in contact with epithelial basement membrane [121].

The genetic ablation of enteric glial cells using transgenic mice expressing herpes simplex virus thymidine kinase from the mouse glial fibrillary acidic protein (GFAP) promoter, performed by Bush et al., showed that when the animals were treated with ganciclovir, an increased inflammatory response ensued and led to death with an underlying severe jejunoileitis [123]. In another study using the same experimental model, Savidge et al. demonstrated that the involvement of EGC with the intestinal barrier function could, at least in part, be due to the release of S-nitrosoglutathione (GSNO), a small molecule that regulates the tight junctions of the epithelial cells. Of notice, GSNO was also shown to be able to restore mucosal barrier function in CD colonic mucosal specimens [124]. In another experimental study focused on EGC, Zhang et al. revealed that glial-derived neurotrophic factor (GDNF), another molecule released by EGC, could also regulate the integrity of the intestinal barrier. Using the dextran sodium sulfate- (DSS-) induced colitis model, the investigators prevented the increase in intestinal permeability and the full inflammatory response, by treating animals with GDNF [125]. Subsequently, another study proposed the existence of an EGC self-protecting mechanism, in which GDNF protects EGC from apoptosis. The authors hypothesized that alterations in such autocrine loop would lead to a defective barrier, mucosal disruption, and development and enhancement of CD inflammation [126]. It is intriguing to note that, in the noninflamed intestinal mucosa of patients with CD, the EGC network was particularly disrupted [127] corroborating the idea that the loss or decrease of EGC might contribute to the pathogenesis of IBD.

8. Involvement of Intestinal Microbiota in the ENS

Trillions of bacteria colonize the gut, with hundreds of different species unevenly distributed throughout the GI tract, basically shaped by diet and immune and genetic factors of the host [128]. In humans, chronic inflammatory disorders have been associated with abnormalities in the microbiota, which may actively modulate disease phenotypes and behavior [129, 130]. In CD, for example, it has been widely accepted that abnormalities of the gut microbiota are present, where there is either an altered composition of the microbiota or an abnormal immune response against the commensal microbiota, or both [131]. Another study in CD demonstrated an increased abundance in Bacteroidetes and Proteobacteria in contrast to a decrease in Firmicutes [132] and probably more importantly a reduction in bacterial diversity [133]. In UC, there is still limited evidence for a major pathogenic role of the microbiota, but dysbiosis has also been reported [134].

In virtue of the potential ability of microorganisms to deregulate the physiological equilibrium, innate immune system is the first to respond to microbiota antigens through the recognition of microbial associated molecular patterns (MAMPs) at the transmembrane or cytosolic receptors, known as pattern recognition receptors (PRRs) [135, 136]. These receptors comprise three distinct families: toll-like receptors (TLRs), the nucleotide oligomerization domain- (NOD-) like receptors (NLRs), and retinoic acid inducible gene I- (RIG-I-) like receptors (NLRs). The best-characterized PRR in mammals is the TLRs family [137]. The TLRs are transmembrane proteins that can be expressed in different sites of the gut [138, 139] and also in components of the nervous system [140]. They trigger the activation of nuclear factor kappa-B (NFkB) through MyD88 and other intracellular mediators, leading to the production of proinflammatory cytokines (as reviewed by Elia et al. [135]). In humans, the enhanced expression of TLR-2 and TLR-4 by crypt epithelial cells demonstrated in active IBD was hypothesized as an indication of a greater ability to respond to distinct bacterial products [141].

Using animal models, Brun et al. found that the absence of TLR2 determines changes in the architecture of the myenteric and submucosal plexuses, leading to bowel dysmotility and

Table 1: Main findings of PAR receptors.

Studies	PARs	Samples	Methods	Results
Corvera et al. (1999) [157]	PAR1 & PAR2	Small intestine of guinea pig	Primary culture RT-PCR	PAR1 and PAR2 are expressed in myenteric neurons that express excitatory and inhibitory neurotransmitters and purinoceptors
Green et al. (2000) [163]	PAR2	Porcine ileum	Immunohistochemistry	Cholinergic and noncholinergic submucosal neurons
Buresi et al. (2005) [164]	PAR1	Mouse colon	RT-PCR Immunohistochemistry	Expressed in full-thickness specimens and mucosal scraping of colon Localized on epithelial cells and on neurons in submucosal ganglia
Ikehara et al. (2010) [165]	PAR1 & PAR2	Mouse cecum	Electrical measurements	PAR1-mediated Cl^- secretion might occur by activation of the receptor on the submucosal secretomotor neurons; PAR2-mediated Cl^- secretion might occur by activation of the receptor on the epithelial cells
Mueller et al. (2011) [166]	PAR1, PAR2, and PAR4	Human submucosal plexus	Voltage- and calcium-sensitive dye recordings	PAR1, rather than PAR2 and PAR4, activates neurons and glia
		Guinea pig (comparative study)	Voltage- and calcium-sensitive dye recordings	PAR2, rather than PAR1 and PAR4, evoked strong responses in enteric neurons and glia

increased susceptibility to intestinal inflammation [142]. In another study analyzing the influence of gut microbiota on the ENS, dysbiosis induced by antibiotics led to local changes in the innate immune system including TLRs and in the expression of sensory-related systems in mice [143]. Of note, results from another experimental study have shown that the exposure of the intestinal interstitium to bacterial cell products can activate nociceptive dorsal root ganglion neurons, leading to production of inflammatory cytokines and increased excitability, directly or independent of TLR signaling [144].

The colonization of the gut by microorganisms starts early in life and is critically important in several functions of the normal GI physiology [145, 146] and also the maturation of the mucosal immune system [147]. For example, in a study with germ-free mice investigating the electrophysiological properties of neurons in the myenteric plexus of the ENS, commensal microbiota was shown to be necessary for normal excitability of gut sensory neurons [148]. Further studies on the subject confirmed that germ-free mice exhibit less excitable intrinsic primary afferent neurons [149], which can be enhanced by the exposure to polysaccharide A [150]. An additional study using germ-free mice corroborated previous findings, demonstrating that the microbiome is crucial for both intrinsic and extrinsic nerve function and gut-brain signaling [151]. Recently, Collins et al. investigated whether the microbiota could influence the postnatal development of the ENS. Investigators found that germ-free mice have a decrease in nerve density and fewer neuronal cell bodies in myenteric ganglia, while in the small bowel, an increased proportion of inhibitory nitrergic neurons was detected. These results appear to support the hypothesis that early exposure to luminal microorganisms is pivotal for the postnatal development of the ENS [152].

Another set of important receptors able to interact with the ENS and modulate neurally mediated intestinal functions is the proteinase-activated receptors (PARs), expressed in different cell types in the gut. PARs belong to a group of G protein-coupled receptors that are activated by proteolytic cleavage [153, 154]. Both PAR1 and PAR2 have been shown to be functional in ENS cells. For instance, it has been demonstrated that PAR2 activation results in depolarization and increased number of action potentials in myenteric [155] and in submucosal neurons [156]. Moreover, PAR1 and PAR2 agonists induce calcium mobilization in myenteric [157] and dorsal root ganglia [158] neurons. In addition to the association with intestinal hyperalgesia and hypersensitivity [159], PAR2 has been shown to actively participate in neurogenic inflammation in the mouse colon [160]. Regarding the potential interaction with the ENS, the release of proteases from activated mast cells, for example, was shown to cleave PAR2 on submucosal neurons, determining acute and long-term hyperexcitability [161] (Figure 2). Interestingly, a recent study has demonstrated that secreted *E. faecalis* proteins, namely, gelatinase, induced permeability in the colonic epithelia of mice, which was absent in PAR2-deficient animals [162]. Together, these results strongly suggest that bacterial enzymes can regulate enteric epithelial permeability and neurogenic inflammation via intestinal PAR. The main findings of PAR receptors are exposed in Table 1. These evidences indicate the existence of interactions between the gut microbiota and the host, with effects on the ENS.

Finally, the accumulating evidence of multidirectional signaling involving the multiple components of the gastrointestinal system, including the bidirectional interplay by which the nervous system modulates the immune response, suggests that these neuronal circuits and neuromediators could be used for novel therapeutic strategies. In addition to gastrointestinal motility, sensitivity, and pain, such therapeutic approach should also provide the possibility of reestablishing immune tolerance and effective controlling chronic intestinal inflammation, for example, through the activation of the vagal anti-inflammatory pathway or the development of new pharmacological agents to control the afferent neuronal signaling.

9. Conclusion

The CNS interacts dynamically with the immune system to modulate inflammation through humoral and neural pathways. Neuroimmune interactions within ENS can modulate gut functions, such as motility, ion transport, and mucosal permeability, contributing to the pathophysiology of several intestinal diseases, including IBD. Intestinal inflammation has been implicated in neuroplasticity, degeneration of the ENS, and alterations in the enteric glia, with an important contribution attributed to oxidative stress. The microbiota also plays a critical role in the intestinal homeostasis and neurogenic inflammation, as it drives postnatal development of the ENS, and affects the intrinsic and extrinsic nerve function and gut-brain signaling. Further investigation of these counterregulatory mechanisms will provide additional insights into neuroimmunomodulation, potentially leading to the identification of novel therapeutic targets for the treatment of inflammatory bowel disorders.

Competing Interests

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

Authors' Contributions

Claudio Bernardazzi and Beatriz Pêgo contributed equally to this work.

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Research Article

Tumor Necrosis Factor Alpha Inhibits L-Type Ca^{2+} Channels in Sensitized Guinea Pig Airway Smooth Muscle through ERK 1/2 Pathway

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Tumor necrosis factor alpha (TNF-α) is a potent proinflammatory cytokine that plays a significant role in the pathogenesis of asthma by inducing hyperresponsiveness and airway remodeling. TNF-α diminishes the L-type voltage dependent Ca^{2+} channel (L-VDCC) current in cardiac myocytes, an observation that seems paradoxical. In guinea pig sensitized tracheas KCl responses were lower than in control tissues. Serum from sensitized animals (Ser-S) induced the same phenomenon. In tracheal myocytes from nonsensitized (NS) and sensitized (S) guinea pigs, an L-VDCC current (ICa) was observed and diminished by Ser-S. The same decrease was detected in NS myocytes incubated with TNF-α, pointing out that this cytokine might be present in Ser-S. We observed that a small-molecule inhibitor of TNF-α (SMI-TNF) and a TNF-α receptor 1 (TNFR1) antagonist (WP9QY) reversed ICa decrease induced by Ser-S in NS myocytes, confirming the former hypothesis. U0126 (a blocker of ERK 1/2 kinase) also reverted the decrease in ICa. Neither cycloheximide (a protein synthesis inhibitor) nor actinomycin D (a transcription inhibitor) showed any effect on the TNF-α-induced ICa reduction. We found that $Ca_V1.2$ and $Ca_V1.3$ mRNA and proteins were expressed in tracheal myocytes and that sensitization did not modify them. In cardiac myocytes, ERK 1/2 phosphorylates two sites of the L-VDCC, augmenting or decreasing ICa; we postulate that, in guinea pig tracheal smooth muscle, TNF-α diminishes ICa probably by phosphorylating the L-VDCC site that reduces its activity through the ERK1/2 MAP kinase pathway.

1. Introduction

Tumor necrosis factor alpha (TNF-α) has been characterized as a potent proinflammatory cytokine that plays a significant role in the pathogenesis of asthma [1–3]. Furthermore, in the sputum and in the bronchoalveolar lavage fluid of asthmatic patients, this cytokine concentration was increased [4, 5]. It has also been reported that, in plasma and bronchoalveolar lavage fluid from sensitized guinea pigs, TNF-α was significantly augmented [6]. Multiple sources point out that, in the airways, TNF-α produces many different effects: it enhances airway smooth muscle contractile response to different

agonists (carbachol, histamine, and bradykinin), it augments agonist-induced Ca^{2+} transients, it diminishes the relaxation induced by isoproterenol, and it favors airway remodeling. All these events contribute to the airway hyperresponsiveness development [7–10].

It is well known that L-type voltage dependent Ca^{2+} channel (L-VDCC) by itself plays a minor role in the agonists-induced airway smooth muscle contraction [11, 12]. Recently, we confirmed that this channel mainly provides extracellular Ca^{2+} to refill the sarcoplasmic reticulum (SR), probably favoring agonists-induced contractile responses [12]. However, in the eighties, its participation in the pathogenesis of asthma was considered controversial since the use of L-VDCC blockers showed great variability in its effects during the treatment of this disease [13], and this inconsistency was never totally clarified. Nowadays, many aspects of inflammation have been thoroughly investigated and it is known that proinflammatory cytokines such as TNF-α alter L-VDCC function in rat cardiac myocytes. In this regard, it has been demonstrated that it reduces, in a reversible manner, the L-VDCC current (ICa) in these cells through the activation of TNF-α receptor 1 (TNFR1) [14, 15]. This TNF-α induced alteration of the L-VDCC function might be happening in airway smooth muscle and could explain the great variability of the L-VDCC blockers effects seen in asthmatic patients.

TNF-α responses in airway smooth muscle have been documented to be mediated by two receptor subtypes, TNFR1 and TNFR2 (also known as p55TNFR and p75TNFR) [16]. TNFR1 activation has been related to augmented agonist-induced Ca^{2+} transients, airway smooth muscle proliferation through modulation of cell mitogenesis [7], upregulation of G proteins (G_i, G_q) [17], and molecules associated with sarcoplasmic reticulum (SR) Ca^{2+} handling such as CD38/cyclic ADP-ribose [18]; all these effects promote airway hyperresponsiveness [19]. Additionally, by activating TNFR1 in airway smooth muscle, TNF-α triggers extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinases (MAPKs) signaling pathway and transcription factors to turn on a variety of genes (interleukins) that mediate inflammatory and immune responses [16]. Meanwhile, through TNFR2, this cytokine activates c-Jun N-terminal kinase (JNK), but not MAPK or p38 MAPKs signaling pathways, and its function is linked to enhance apoptotic cell death [20].

For a long time, in airway smooth muscle, the L-VDCC was characterized through pharmacological and electrophysiological methods [11, 21]. Nevertheless, Du et al. [22] claimed that, using molecular assays, they found all subunits of this channel ($Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$) in the rat bronchial smooth muscle.

Therefore, the aim of the present work was to explore the role of TNF-α on the functionality of the guinea pig airway smooth muscle L-VDCC and define the signaling pathway induced by this cytokine that could be acting on the channel. Additionally, we explored which subunits of the L-VDCC are present in this tissue and if they were modified by sensitization.

2. Material and Methods

2.1. Experimental Animals. Hartley male guinea pigs weighing 400–600 g from our institutional animal facilities (filtered conditioned air, 21 ± 1°C, 50–70% humidity, sterilized bed) fed with Harlan® pellets and sterilized water were used. The experimental protocol was approved by the Scientific and Bioethics Committees of the Facultad de Medicina, Universidad Nacional Autónoma de México (061/215). The experimental protocol closely followed the Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training published by the American Physiological Society. Mexican National Protection Laws on Animal Protection and the General Health Law Related to Health Research (NOM-062-Z00-1999) were also considered.

2.2. Sensitization Procedure. Male guinea pigs were sensitized as described elsewhere [23]. Briefly, at day 0, animals weighing ~250 g received an i.p. and s.c. administration of 500 μg ovalbumin (OA) and 500 μg $Al(OH)_3$ in 0.5 mL saline (0.9% NaCl). At day 8, they were nebulized during 80 sec with 15 mg/mL OA in saline delivered by an ultrasonic nebulizer (model WH-200, Guangdong Yuehua Medical Instruments Factory Co., Ltd., China) and again at day 15 with 1 mg/mL OA in saline during 10 sec. All animals were studied at days 21–25 of sensitization.

2.3. Organ Baths. Nonsensitized (NS) and sensitized (S) guinea pigs were anesthetized with pentobarbital sodium (35 mg/kg, i.p.) and exsanguinated. Eight rings were obtained from tracheas cleaned of connective tissue, and each ring was hung in a 5 mL organ bath chamber containing Krebs solution (in mM): 118 NaCl, 25 $NaHCO_3$, 4.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, and 2 $CaCl_2$ at 37°C. A mixture of 5% CO_2 and 95% oxygen was used to bubble the tissue continuously and maintain the pH at 7.4. To block prostanoids formation, indomethacin (1 μM) was added to the Krebs solution. Tension developed by the tissue was registered by an isometric force transducer (model FT03; Grass Instruments, West Warwick, RI, USA) connected to a signal conditioner (CyberAmp 380, Axon Instruments, Foster City, CA, USA) and to an analog-to-digital interface (Digidata 1440A; Axon Instruments). Data were recorded and analyzed with an AxoScope version 10.2 software (Axon Instruments).

Tracheal rings were submitted to a resting tension of 1 g during 30 min. Afterwards they were stimulated three times with KCl (60 mM) to allow tissue conditioning and optimization of the contractile apparatus. The last response to KCl was compared between NS and S tracheal tissues. In another set of experiments, NS tracheal preparations were incubated during 60 min with serum from sensitized animals at different percentage concentrations (V/V, 1, 2.5, 5, and 10%). Afterwards, a cumulative KCl concentration-response curve was done (20, 40, and 60 mM). These responses were expressed as % of the third 60 mM KCl stimulation.

2.4. Patch Clamp Studies. Guinea pig tracheal smooth muscle from NS and S animals was dissected free of epithelium and connective tissue and placed in 5 mL Hanks solution

containing 2 mg L-cysteine and 0.04 U/mL papain. The pH was always adjusted to 7.4 with 1 M $NaHCO_3$ and tissues were then incubated for 10 min at 37°C. The tissues were washed with Leibovitz's solution to remove enzyme excess and afterwards placed in Hanks solution with 1 mg/mL collagenase type I and 0.5 mg/mL protease during 10 min at 37°C. Myocytes were gently dispersed by mechanical agitation until detached cells were observed. Leibovitz's solution was used again to stop enzymatic activity and cells were centrifuged at 600 rpm, 20°C during 5 min, and the supernatant was discarded. This procedure was repeated once.

Tracheal myocytes from NS or S animals were cultured as follows: the cell pellet was resuspended in minimum essential medium containing either 10% fetal bovine serum (FBS), 10% serum from no-sensitized (Ser-NS) animals, or 10% serum from sensitized animals (Ser-S), 2 mM L-glutamine, 10 U/mL penicillin, 10 μg/mL streptomycin, and 15 mM glucose and plated on round cover slips coated with rat tail sterile collagen. Some myocytes cultures from NS animals containing FBS or Ser-S were added with tumor necrosis factor alpha (TNF-α, 20, 200, or 1000 μg/L; these concentrations were used previously to block L-VDCC in rat cardiac myocytes [15]), a small-molecule inhibitor of TNF-α (SMI-TNF, 32 μM; at this concentration, it promotes subunit disassembly of TNF-α and inhibits its activity [24]), the TNF-α receptor 1 (TNFR1) antagonist (WP9QY, 3.2 or 10 μM [25]), or an inhibitor of ERK 1/2 (U0126, 5 μM [26]). We used U0126 since it has been reported that TNF-α effects in airway smooth muscle are, in part, through ERK 1/2 MAPKs signaling pathway [26]. Cells were then cultured at 37°C in a 5% CO_2 in oxygen during 48 h. In another set of experiments, NS myocytes were cultured with FBS during 24 h for them to adhere to the surface of the round cover slips. Afterwards, they were incubated with 1000 μg/L TNF-α or TNF-α with the protein synthesis inhibitor cycloheximide 20 μM [27] or TNF-α with the transcription inhibitor actinomycin D 3 μM [28] during further 24 h.

Subsequently, myocytes on the cover glass were placed at the bottom of the 0.7 mL perfusion chamber and allowed to settle down. The chamber was perfused by gravity (~1.5–2.0 mL/min) with an external solution containing Ba^{2+} to replace Ca^{2+} as the inward charge carrier to measure Ca^{2+} currents and in mM 136 NaCl, 6 CsCl, 5 $BaCl_2$, 11 glucose, 10 HEPES, and 0.1 niflumic acid, pH 7.4 adjusted with CsOH. All experiments were performed at room temperature (~21°C).

To record Ca^{2+} currents activated by depolarizing voltage steps (i.e., voltage clamp) through an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA), the standard whole-cell configuration was used. Patch pipettes were made with 1B200F-6 glass (Word Precision Instruments, Sarasota, FL, USA) using a horizontal micropipette puller (P-87, Sutter Instruments Co, Novato, CA). Each pipette had a resistance ranging from 2 to 4 MΩ. The internal solution consisted of (mM) 130 CsCl, 2 $MgCl_2$, 10 HEPES, 10 EGTA, 3.6 ATP disodium salt, and 1.9 GTP sodium salt, pH 7.3, adjusted with CsOH. Currents were filtered at 1–5 KHz, digitized (Digidata 1440A, Axon) at 10 KHz, stored, and analyzed in a computer through specialized software (pClamp v10.2, Axon).

Tracheal myocytes showed Ca^{2+} currents when subjected to series of conditioning hyperpolarizing and depolarizing pulses of potentials ranging from −60 to +50 mV in 10 mV increments from a holding potential of −60 mV during 100 ms, 1 Hz. Changes in the currents from the protocols described above were evaluated as maximal current peak to each voltage tested.

2.5. Total RNA Extraction and RT-PCR. Total RNA was purified using the RNeasy Mini Kit (Qiagen, CA, USA), to prevent DNA contamination; DNase Set digestion (Qiagen) was used and eluted with RNAse-free water. Concentration of total RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Barrington, IL, USA). RNA purity was evaluated by the absorbance ratios at 260 : 230 nm and 260 : 280 nm, where a 1.8 ratio was suitable for proceeding with cDNA synthesis. Total RNA was reverse-transcribed to cDNA using transcriptor reverse transcriptase (Roche, Life Science, IN, USA), random primer (Invitrogen, Life Technologies, CA, USA), and 1 μg of total RNA. The oligonucleotides were designed based on the sequences reported in the NCBI database. The oligonucleotide sequence, PCR melting temperature (MT), and accession number of the different subunits of the L-VDCC ($Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$) are shown in Table 1. Negative controls were prepared without template and with 50 ng of each total RNA extracted. The amplification conditions were 5 min at 94°C, followed by 1 min at 94°C, 1 min to MT (see Table 1) and 1 min at 72°C, and a final extension of 10 min. The reactions were performed by duplicate. The PCR products were electrophoresed in a 1.5% agarose gel with GelRed (Biotium, CA, USA). The PCR products corresponded to the estimated length. Amplicon identity was corroborated by sequencing. Images were digitized using Typhoon FLA 9500 laser scanner (GE Healthcare, CT, USA).

2.6. Double Immunofluorescence. Tracheal tissues were fixed in 4% paraformaldehyde in sodium phosphate buffer (PBS), overnight at 4°C, and dehydrated with ascendant series of ethanol until their inclusion in paraffin. The tissue blocks were cut in 10 μm slices. Paraffin was removed by incubation in xylol, followed by graded alcohols. Heat induced antigen retrieval was performed by placing slides in a pressure cooker (Biocare Medical, CA, USA) in 1x Diva Decloaker (Biocare Medical). Slices were transferred into PBS and permeabilized with 0.05% Tween-20 in PBS. To block nonspecific binding to proteins, 10% horse serum was applied on the slices for two h. The slices were incubated with the primary antibodies to $Ca_V1.2$ and $Ca_V1.3$ (subunits of L-VDCC, Alomone Labs., Cat. numbers ACC-003 and ACC-311, resp., Jerusalem, Israel), both antibodies at a dilution 1 : 50, overnight at 4°C. The secondary antibody Alexa488 donkey anti-rabbit IgG (Life Technologies, CA, USA) was incubated (1 : 200) for 30 min. The slices were incubated with the next primary antibody, α-actin (Santa Cruz Biotechnology, Cat. number sc-58669, TX, USA), and Alexa Fluor 555 donkey anti-mouse (Life Technologies) 1 : 400 for 30 min, as the secondary antibody. Tissue sections were kept with fluorescence mounting medium Dako (Dako, North America Inc., CA, USA).

Table 1: Sequence of primers and PCR conditions for the different subunits of L-VDCC.

Gene	Oligonucleotides 5′-3′	Length (bp)	Amplification conditions	Reference
$Ca_V1.1$	Fw: TGGTACGTCGTCACCTCCT Rv: CATCTATGATGCTGCCGATG	237	MT = 56°C 34 cycles	XM_013158049
$Ca_V1.2$	Fw: AATTGCTCTGAAGATGACAGC Rv: AGCTGCCAGGACATTGTCG	471	MT = 56°C 30 cycles	NM_001172923.1
$Ca_V1.3$	Fw: TCCCGCCGGCAGGACTAT Rv: ATCACCTTTAACCTCCCCCCA	459	MT = 56°C 32 cycles	XM_005008263
$Ca_V1.4$	Fw: TACCCATCCCGGGTACCTAT Rv: GAAGTGGGAGAAGATAGACT	436	MT = 56°C 34 cycles	XM_013144287
GAPDH	Fw: TGAAGGTGAAGGTCGGTGTGAACG Rv: CATGTAGGCCATGAGGTCCACCAC	~1000	MT = 56°C 29 cycles	Chávez et al., 2013 [29]

Fw: forward primer, Rv: reverse primer, MT: melting temperature, and bp: base pairs.

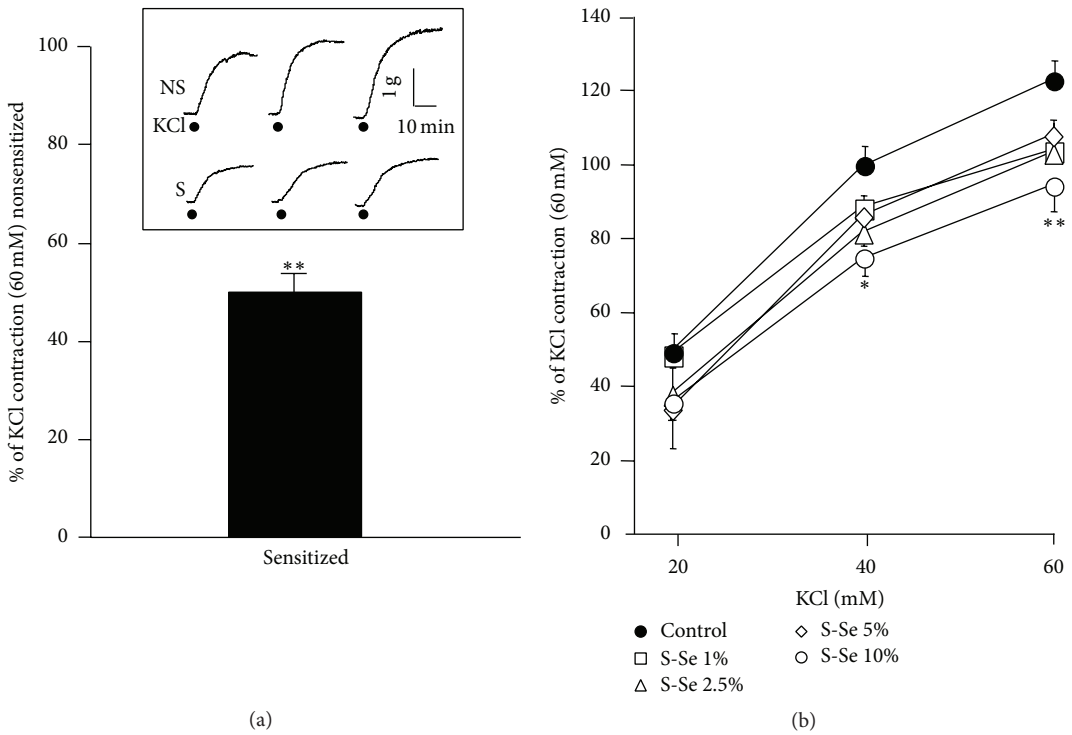

Figure 1: Sensitization diminished KCl-induced contraction in guinea pig tracheas. Responses to 3 consecutive stimulations of 60 mM KCl were higher in tracheal rings from nonsensitized guinea pigs (NS, $n = 14$) than in tissues from sensitized (S, $n = 17$) animals (inset). (a) The third KCl response from NS tissues was considered as 100%, and statistical difference was found when compared with the S group. (b) Nonsensitized tracheas, incubated with different percentage (V/V) concentrations of sensitized guinea pigs serum (Ser-S, $n = 5$, each), showed decreased responses to KCl (20–60 mM), reaching statistical significance only with the highest Ser-S concentration tested. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group. Bars and symbols represent mean ± SEM.

To determine the specificity of immunofluorescence, the antigens for $Ca_V1.2$ and $Ca_V1.3$ were used to saturate the primary antibody. The nuclei were counterstained with DAPI (Life Technologies). The immunofluorescences were observed using a fluorescence microscope (Model Eclipse Ni-U, Nikon, Japan). For display purposes, merged images were constructed in which $Ca_V1.2$ and $Ca_V1.3$ were green, α-actin red, and nuclei blue.

2.7. Drugs and Chemicals. Tumor necrosis factor alpha (TNF-α), U0126 ethanolate, an inhibitor of ERK 1/2 kinase (1,4-diamino-2,3-dicyano-1,4-bis-(0-amino-phenylmercapto)butadiene ethanolate), cycloheximide, actinomycin D, and indomethacin were purchased from Sigma Chem. Co. (St. Louis, MO, USA). TNF-α small-molecule inhibitor and WP9QY, an antagonist of the TNF-α receptor 1, were purchased from Calbiochem (Darmstadt, Germany).

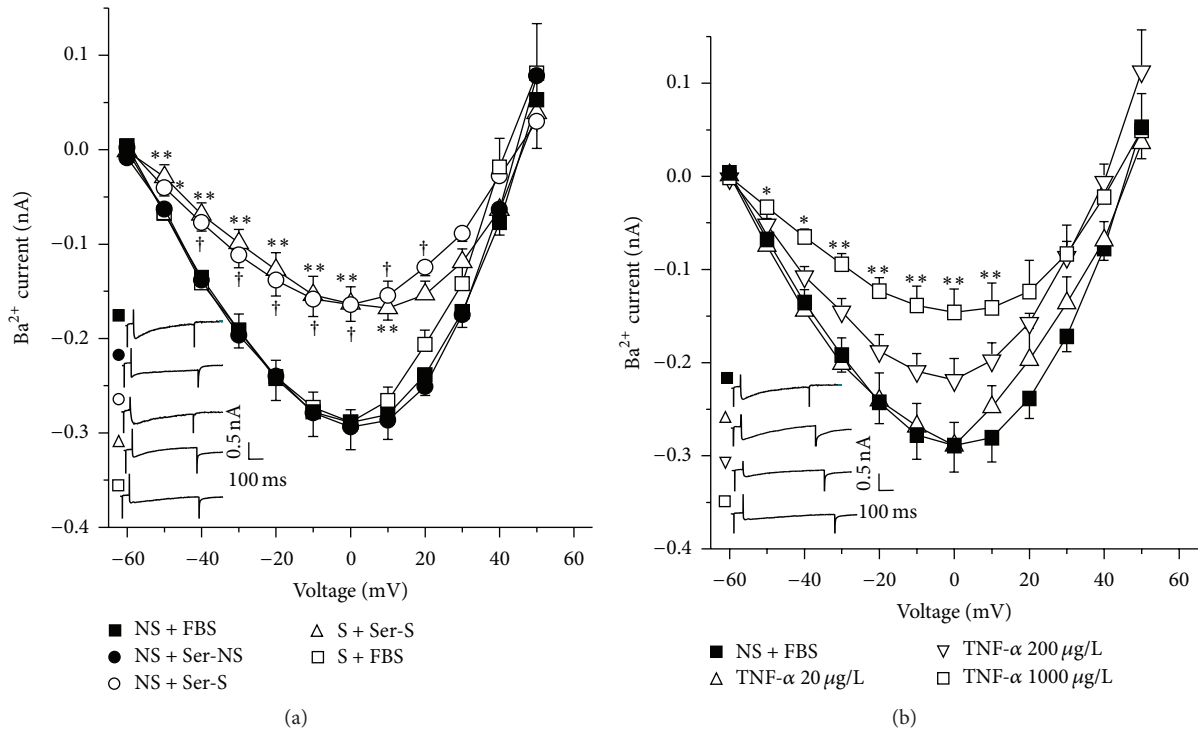

Figure 2: Voltage dependent L-type Ca^{2+} currents were diminished by sensitized guinea pig serum and by TNF-α in guinea pig tracheal myocytes. (a) Similar Ba^{2+} currents, equivalent to Ca^{2+} currents, induced by 10 mV increments were observed in myocytes from nonsensitized (NS) animals grown with fetal bovine serum (FBS) or serum from nonsensitized guinea pigs (Ser-NS). These currents were significantly diminished when myocytes from NS and sensitized (S) animals were grown with serum from sensitized guinea pigs (Ser-S, 10%). This phenomenon was not seen when myocytes from S were grown with FBS ($n = 9$ for each group). (b) Ba^{2+} currents in myocytes from NS grown with FBS showed a decrease when TNF-α was added. These responses were concentration dependent and only the highest (1000 μg/L) produced a significant reduction of this current ($n = 5$ for each group). Insets in each figure represent original recordings. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{\dagger}p < 0.01$ when compared with NS + FBS group. Symbols represent mean ± SEM.

2.8. Statistical Analysis. Data values obtained in organ baths and RT-PCR experiments were analyzed through nonpaired Student's t-test or one-way analysis of variance followed by Dunnett's multiple comparison tests, as well as the results for the patch clamp experiments. Along the paper and figures, data are expressed as mean ± SEM. In patch clamp experiments, each cell belongs to a different animal. Statistical significance was set at $p < 0.05$ bimarginally.

3. Results

3.1. Sensitization Reduced KCl Responses in Tracheal Smooth Muscle from Guinea Pig. In tracheal rings from NS guinea pigs, KCl induced a contraction that was significantly higher than the response observed in preparations from S animals (Figure 1(a)). NS tracheas, incubated with different percentage concentrations of sensitized guinea pigs serum (Ser-S, 1, 2.5, 5, and 10%, $n = 5$) showed decreased responses to cumulative concentrations of KCl (20, 40, and 60 mM), reaching statistical significance only with the highest Ser-S concentration tested (Figure 1(b)).

3.2. Possible Role of TNF-α in the Decrease of Ca^{2+} Currents Induced by Serum from Sensitized Animals in Tracheal Myocytes from Guinea Pig. In the voltage clamp experiments with single myocytes from NS animals grown with fetal bovine serum (FBS), step depolarizations from −60 to 50 mV from a holding potential of −60 mV produced a voltage dependent inward Ca^{2+} current (ICa). The peak inward current reached maximal amplitude at 0 mV. This current was corroborated to be from the L-type Ca^{2+} channels, because it was almost abolished (86.35 ± 1.42%, data not shown) by 1 μM nifedipine ($n = 4$), an L-type Ca^{2+} channel blocker. A similar ICa was observed in myocytes from NS guinea pigs cultivated with serum from NS animals (Ser-NS). When NS myocytes were grown with serum from sensitized guinea pigs (Ser-S) ICa was significantly diminished and this effect was also noticed in cells from S animals incubated with Ser-S. This last effect was not seen when S myocytes were cultured with FBS (Figure 2(a)). NS myocytes grown with different TNF-α concentrations showed a concentration dependent decrease of ICa, reaching only statistical significance to the highest concentration tested (1000 μg/L, Figure 2(b)). These findings suggest that Ser-S contains some chemical mediator responsible for this ICa decrement, possibly TNF-α. This hypothesis was confirmed by using a small-molecule inhibitor of TNF-α (SMI-TNF) and a TNF-α receptor 1 (TNFR1) antagonist (WP9QY). We found that SMI-TNF

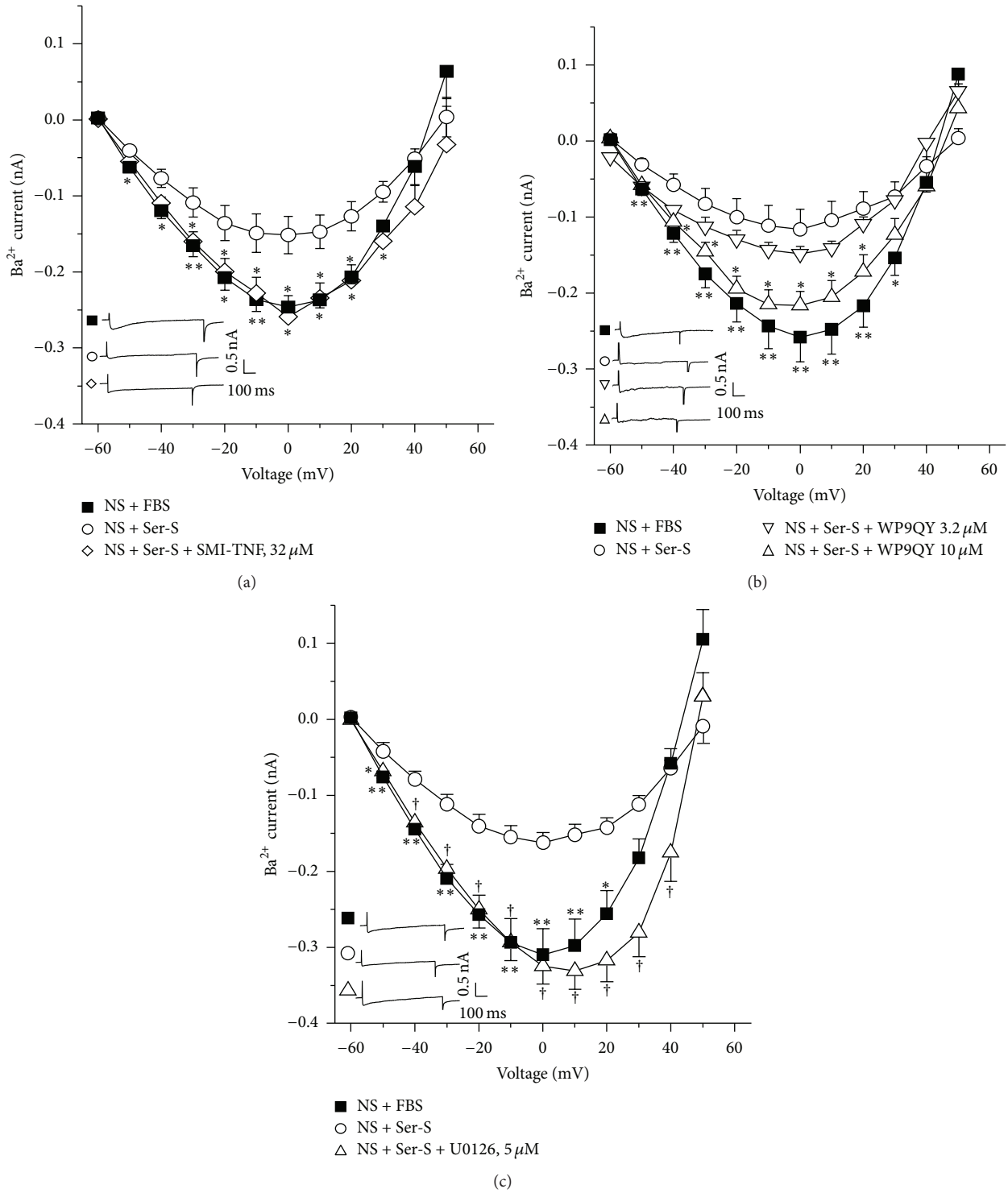

FIGURE 3: TNF-α diminishes the L-type Ca^{2+} currents through activation of the TNF-α receptor 1 (TNFR1) and MAP kinase signaling pathway in guinea pig tracheal myocytes. (a) Myocytes from nonsensitized animals (NS) grown with fetal bovine serum (FBS) showed an inward Ba^{2+} current. This current was reduced when NS myocytes were grown with serum from sensitized guinea pig (Ser-S, 10%). This decrease was abolished when the small-molecule inhibitor of TNF-α (SMI-TNF, $n = 7$), which impedes this cytokine from binding to its receptor, was incubated during cell growth. (b) The Ba^{2+} current diminution induced by Ser-S incubation in NS myocytes was also reversed, in a concentration dependent manner, by the TNFR1 antagonist (WP9QY, $n = 9$). (c) The Ba^{2+} current decrease induced by Ser-S was abolished when myocytes were incubated with an inhibitor of ERK 1/2 kinase (U0126, $n = 6$). Insets in each figure represent original recordings. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\dagger}p < 0.01$ when compared with NS + Ser-S group. Symbols represent mean ± SEM.

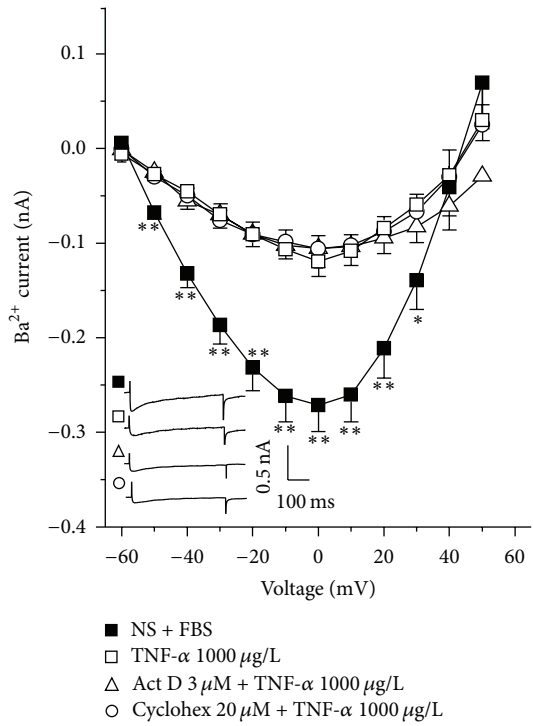

Figure 4: The Ba^{2+} current reduction induced by TNF-α is not mediated by a synthetic pathway activation in guinea pig tracheal myocytes. Ba^{2+} current evoked by step depolarization from −60 to 50 mV in tracheal cells from nonsensitized guinea pigs added with fetal bovine serum (NS + FBS, $n = 7$) were significantly diminished when myocytes were grown with TNF-α ($n = 7$). Neither actinomycin D (Act D, $n = 6$) nor cycloheximide (Cyclohex, $n = 9$) addition during myocyte growth altered TNF-α induced effect on the Ba^{2+} current. Inset represents original recordings. $^{*}p < 0.05$, $^{**}p < 0.01$, when compared with TNF-α group. Symbols represent mean ± SEM.

completely abolished the ICa decrease induced by Ser-S in NS myocytes; in this experimental protocol, the current values observed were equal to those reached by the control group (NS + FBS, Figure 3(a)). In this regard, WP9QY showed a concentration dependent effect on the Ser-S induced ICa decrement and only the highest concentration tested reached statistical significance (10 μM, Figure 3(b)). The exploration of TNFR1 signaling pathway by blocking the ERK 1/2 kinase with U0126, also showed a reversal of the decrease in ICa induced by Ser-S in NS myocytes (Figure 3(c)). Neither cycloheximide nor actinomycin D showed any effect on the TNF-α-induced ICa reduction, demonstrating that this response is not related to a synthetic pathway (Figure 4).

3.3. L-VDCC Subunits $Ca_V1.2$ and $Ca_V1.3$ Are Expressed in Guinea Pig Airway Smooth Muscle. In guinea pig airway smooth muscle from NS animals, we found that the main mRNA was for $Ca_V1.2$ followed by $Ca_V1.3$. The mRNA for $Ca_V1.1$ and $Ca_V1.4$ were not expressed in this tissue. Smooth muscle from S animals showed a similar expression pattern in the Ca_V subunits mRNA, and these results were not different from NS tissues (Figure 5). We corroborated that the primers used for $Ca_V1.1$ and $Ca_V1.4$ were adequate by testing them in positive control tissues: skeletal muscle for $Ca_V1.1$ and retina for $Ca_V1.4$ (Figure 5). Since the mRNA for $Ca_V1.2$ and $Ca_V1.3$ were the main subunits found in guinea pig airway smooth muscle, we performed immunofluorescence for $Ca_V1.2$ and $Ca_V1.3$ proteins in both NS and S tissues. We found that $Ca_V1.2$ and $Ca_V1.3$ are present in NS and S guinea pig airway smooth muscle and also in the epithelium and cilia. Negative controls carried out by the incubation of the respective blocking peptide showed no staining (Figures 6(i) and 7(i)).

4. Discussion

In the present study we found that S guinea pig tracheas had a lower contractile response to KCl when compared to NS tissues. This finding was mimicked when NS tracheas were incubated with serum from sensitized animals (Ser-S). Additionally, ICa from NS or S myocytes was reduced when cells were grown with Ser-S and this effect was not seen when S cells were grown with FBS pointing out that some inhibitory factor was present in the Ser-S. This ICa decrease was also observed when NS myocytes cultured with FBS were added with TNF-α (Figure 2(b)). We corroborated that the chemical mediator present in Ser-S was TNF-α because the SMI-TNF and the TNFR1 antagonist abolished the ICa decrease induced by Ser-S. Additionally, the ERK 1/2 kinase inhibitor also reversed the ICa reduction induced by Ser-S implying a MAP kinase-dependent pathway. Furthermore, the main subunits of the L-VDCC expressed in guinea pig airway smooth muscle were $Ca_V1.2$ and $Ca_V1.3$, and sensitization did not modify their expression.

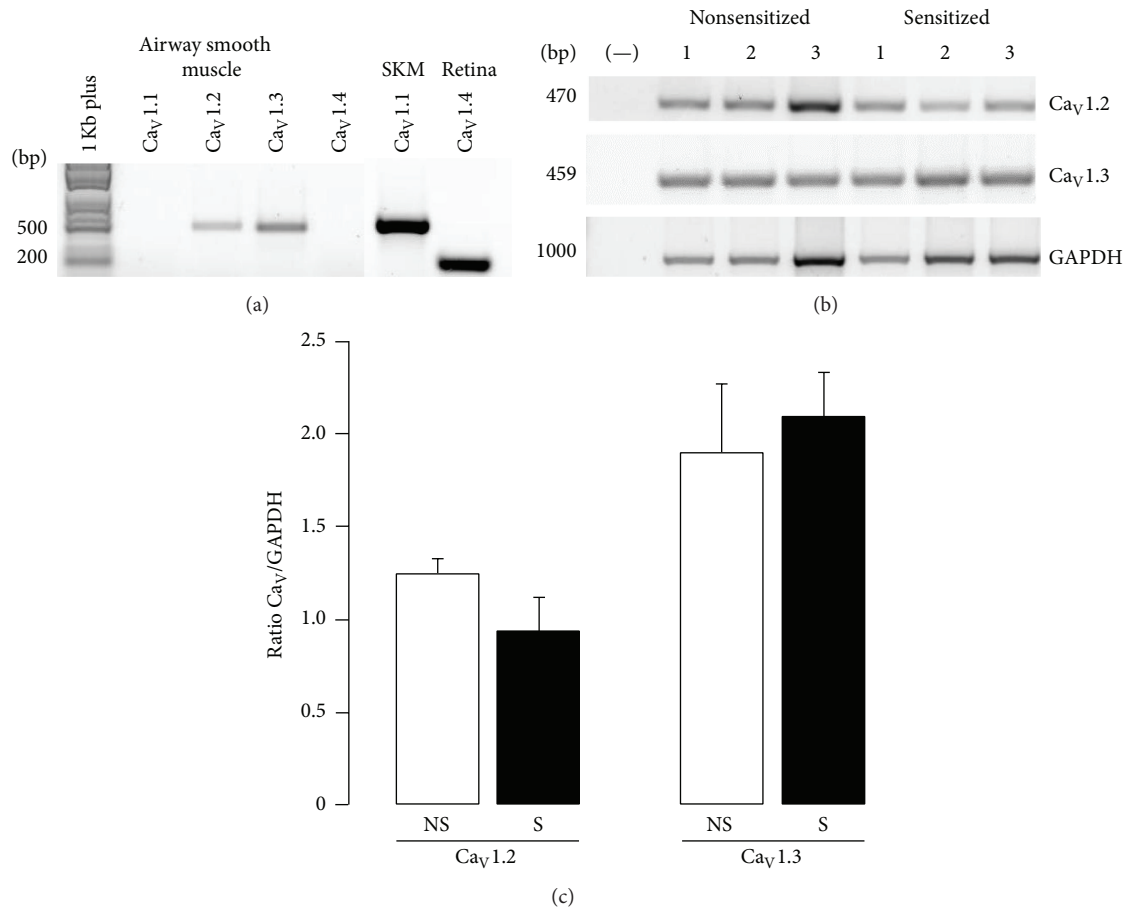

Figure 5: Detection of mRNA for L-VDCC subunits in guinea pig tracheal smooth muscle, as revealed by RT-PCR. (a) In airway smooth muscle, the PCR products at 470 and 459 bp length correspond to $Ca_V1.2$ and $Ca_V1.3$ cDNA, respectively. In this tissue, $Ca_V1.1$ and $Ca_V1.4$ were not found. Positive controls for these subunits were skeletal muscle (SKM, ~500 bp) and retina (~200 bp). Lane at the left corresponds to 1 Kb Plus DNA Ladder. (b) Representative PCR blots for $Ca_V1.2$ and $Ca_V1.3$ from nonsensitized (NS, $n = 3$) and sensitized (S, $n = 4$) smooth muscles. The first column in each blot corresponds to a negative control without template. The lower panel displays constitutive cDNA of GAPDH. (c) Densitometry data analysis for mRNA from $Ca_V1.2$ and $Ca_V1.3$ subunits showing no statistical significance between NS and S. Bars correspond to mean ± SEM.

We recently found that, in guinea pig airway smooth muscle, L-VDCC and store operated Ca^{2+} channels (SOC, capacitative Ca^{2+} entry) are the main membrane Ca^{2+} handling proteins involved in providing extracellular Ca^{2+} for SR Ca^{2+} refilling to sustain contraction [12]. Additionally, former works in porcine and human airway smooth muscle claimed that L-VDCC and receptor operated Ca^{2+} channels (ROC) were the main channels involved in this mechanism [30, 31]. In this context, each of these channels only partially mediates the sustained contraction, although L-VDCC seems to participate to a lesser extent than SOC. Interestingly, when both channels (L-VDCC and SOC) were consecutively blocked, a potentiation effect was seen [32].

The inflammatory condition developed by sensitization induced that, in guinea pig tracheas, KCl responses were notably diminished and this effect was reproduced by Ser-S. Because TNF-α concentration has been demonstrated to be increased in plasma and bronchoalveolar lavage fluid from sensitized guinea pigs [6], we hypothesise that this cytokine was responsible for this decreased response. This was confirmed when TNF-α diminished ICa in single NS tracheal myocytes in the same way as Ser-S (Figure 2). These findings seem paradoxical, since it is recognized that TNF-α, a proinflammatory cytokine, alters normal airway myocytes to a hyperreactive state. In this context, in human airway smooth muscle, this cytokine induces an increment in the capacitative Ca^{2+} entry due to an increased expression of STIM1 an Orai1 [33, 34]. Furthermore, in this tissue TNF-α also causes an upregulation of CD38 expression, a cell surface protein that regulates the synthesis and degradation of cyclic ADP-ribose (cADPR) [35]. This molecule provokes Ca^{2+} release from the SR through stimulation of the ryanodine receptor. The overexpression of CD38 could augment cADPR and SR Ca^{2+} release favoring airway hyperresponsiveness. Moreover, the sarcoplasmic reticulum ATPase (SERCA) expression in human airway smooth muscle exposed to TNF-α was decreased [36]. All the abovementioned evidences point out that TNF-α augments cytosolic Ca^{2+} to promote

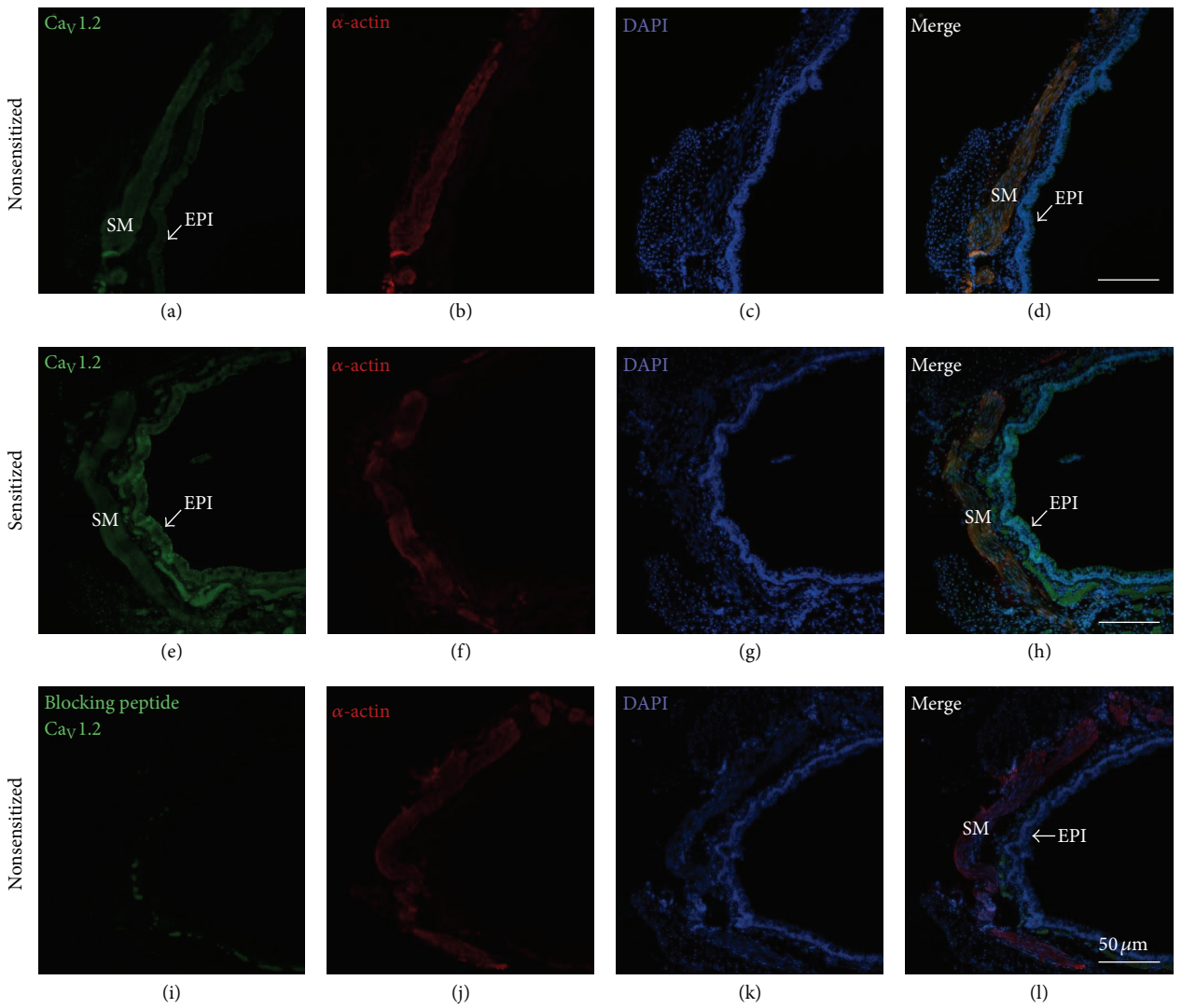

FIGURE 6: Immunofluorescence for $Ca_V1.2$ in nonsensitized and sensitized guinea pig tracheal smooth muscle. The first column shows immunoreactivity for $Ca_V1.2$ (stained green) in nonsensitized (a) and sensitized tissues (e); notice that $Ca_V1.2$ is located in the airway smooth muscle (SM) and epithelium (EPI, pointed by arrow); blocking peptide completely eliminated the fluorescence (i). The second and the third columns illustrate smooth muscle α-actin (stained red; (b), (f), (j)) and cell nuclei (DAPI, stained blue; (c), (g), (k)). The last column depicts merged images of the former three columns ((d), (h), (l)). In these merged images, $Ca_V1.2$ is seen to be colocalized with α-actin (stained yellow) on the smooth muscle.

airway hyperresponsiveness. Because these mechanisms that increase cytosolic Ca^{2+} concentration are upgraded by TNF-α, the need for further Ca^{2+} entry through L-VDCC could be less; therefore it seems conceivable that this cytokine might induce a reduced ICa as compensatory effect.

We found that the subunits of L-VDCC in guinea pig tracheal smooth muscle were $Ca_V1.2$ and $Ca_V1.3$ and that their expression was not modified by sensitization. Thus, the ICa observed in this tissue mainly corresponds to these subunits. Therefore, the ICa decrease induced by TNF-α was related to L-VDCC function and not to a reduction in their expression during sensitization (Figures 5–7).

It is well known that TNF-α receptors are coupled to a mitogen-activated protein (MAP) kinases cascade involving either ERK, JNK, or p38 MAPKs to induce several transcription factors that control gene expression [37]. This MAP kinase signaling pathway usually induces gene expression in nonmuscle cells, while, additional to gene expression in airway smooth muscle cells, muscarinic M_2 receptor stimulation leads to caldesmon phosphorylation through ERK MAP kinases [38]; when it is in its nonphosphorylated state, caldesmon inhibits the actomyosin ATPase and reduces smooth muscle force production [39]. Thus, TNF-α effect on L-VDCC in our tissue could be due either to a phosphorylation mechanism or to a synthetic pathway.

In airway smooth muscle TNF-α exerts its actions by activating TNFR1 and TNFR2 receptors. At least the upregulation of CD38 expression induced by this cytokine has

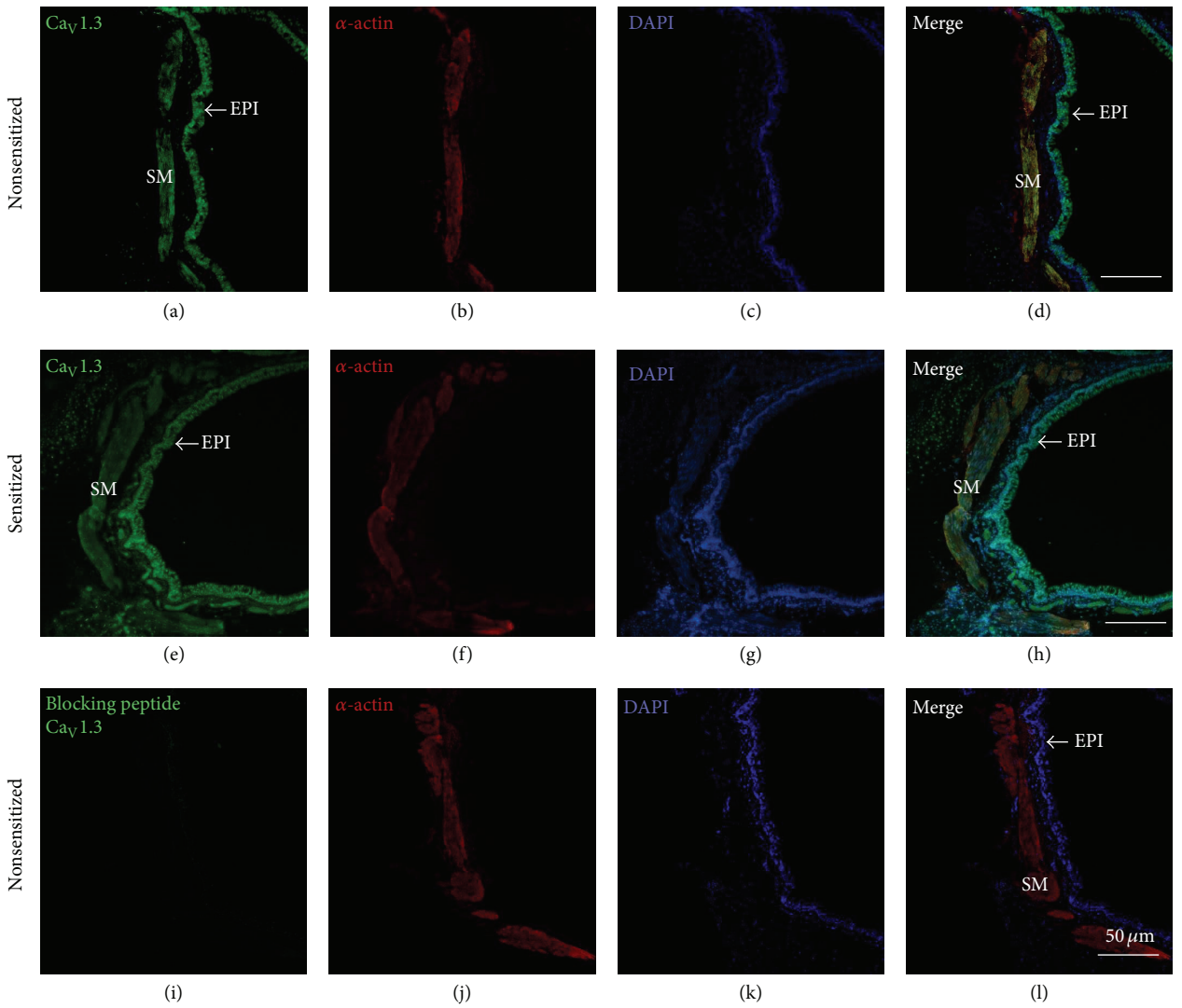

FIGURE 7: Immunofluorescence for $Ca_V1.3$ in nonsensitized and sensitized guinea pig tracheal smooth muscle. The first column shows immunoreactivity for $Ca_V1.3$ (stained green) in nonsensitized (a) and sensitized tissues (e); notice that $Ca_V1.3$ is located in the airway smooth muscle (SM) and epithelium (EPI, pointed by arrows); blocking peptide completely eliminated the fluorescence (i). The second and the third columns illustrate smooth muscle α-actin (stained red; (b), (f), (j)) and cell nuclei (DAPI, stained blue; (c), (g), (k)). The last column depicts merged images of the former three columns ((d), (h), (l)). In these merged images, $Ca_V1.3$ is seen to be colocalized with α-actin (stained yellow) on the smooth muscle.

been confirmed to be mediated by TNFR1 activation of downstream ERK and p38 MAP kinase signaling pathway without involving NF-κB nor AP-1 nuclear transcription factors [18]. In NS myocytes from guinea pig tracheas, the presence of Ser-S induced an ICa reduction that was abolished by a small-molecule inhibitor of TNF-α, confirming that this cytokine is responsible for this effect; TNF-α induced the same ICa decrease as Ser-S. Furthermore, we corroborated that the receptor involved in this ICa reduction was TNFR1, because WP9QY, an antagonist of this receptor, reversed the ICa diminution. Therefore, we verified that ERK signaling pathway was involved in this ICa reduction, since U0126 completely abolished this effect. Because this signaling pathway usually turns on transcription factors and therefore protein synthesis, we confirmed that this was not the case; neither actinomycin, a transcription inhibitor, nor cycloheximide, a protein synthesis inhibitor, had any effect on the ICa decrease induced by TNF-α. Thus, our results point out that this cytokine effect is probably related to a phosphorylation process of the L-VDCC through ERK 1/2 MAP kinase. In this regard, ERK 1/2 MAP kinase has been implicated in phosphorylating rat ventricular myocytes L-VDCC in two sites: β_2 Ser^{496} and α_1 Ser^{1928}. Phosphorylation of the former site may be linked to downregulation of the L-VDCC activity, while the second site's phosphorylation may lead to upregulation of the function [40]. Therefore, TNF-α activation of MAP kinase pathway may be phosphorylating the L-VDCC in β_2 Ser^{496} to reduce the ICa in guinea pig airway smooth muscle (Figure 8), although further research is required.

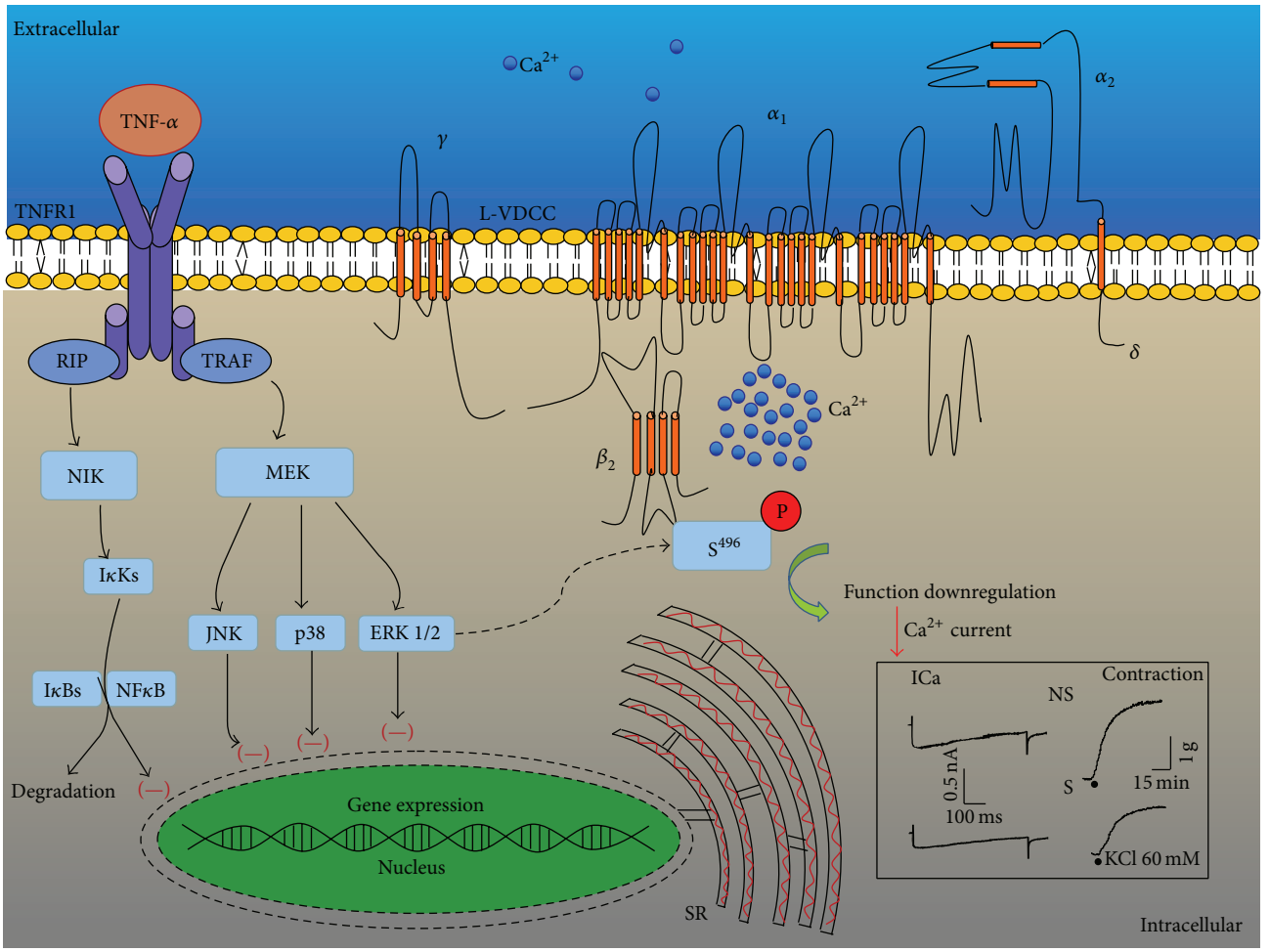

Figure 8: Schematic representation of the proposed mode of action of tumor necrosis factor α (TNF-α) on the L-type voltage dependent calcium channel (L-VDCC) of the guinea pig airway smooth muscle. TNF-α whether exogenous or present in sensitized guinea pig serum (Ser-S) activates its receptor 1 (TNFR1). Afterwards, it promotes synthetic signaling pathways: it activates receptor interacting protein (RIP), NF-κB-inducing kinase (NIK), and IκB kinases (IκKs) that phosphorylate NFκB inhibitors (IκBs) activating nuclear factor κB (NFκB) and through TNF receptor-associated factor (TRAF), map kinase kinase (MEK) and extracellular signal-regulated kinase (ERK 1/2), p38 MAPK, or c-Jun N-terminal kinase (JNK). We demonstrated that a synthetic pathway was not responsible for the diminution in the Ca^{2+} current (ICa, see Figure 4). Nevertheless, ERK 1/2 might be directly phosphorylating serine^{496} (S^{496}) on the β_2 subunit of the L-VDCC, favoring a downregulation of the ICa. Inset illustrates ICa in tracheal myocytes and contraction in tracheal rings from guinea pigs. NS implies nonsensitized tissues or cells and this indicates the absence of exogenous TNF-α or serum from Ser-S. S illustrates original recordings from sensitized tissues or cells in the presence of Ser-S; notice that both ICa and contraction are diminished. Other subunits of the L-VDCC: α_1, α_2, γ, δ. SR: sarcoplasmic reticulum.

Abbreviations

TNF-α:	Tumor necrosis factor alpha
L-VDCC:	L-type voltage dependent calcium channel
Ser-S:	Serum from sensitized animals
NS:	Nonsensitized guinea pig
S:	Sensitized guinea pig
ICa:	Voltage dependent inward calcium current
SMI-TNF:	Small-molecule inhibitor of TNF alpha
TNFR1:	Tumor necrosis factor receptor 1
WP9QY:	TNF alpha receptor 1 antagonist
$Ca_V1.2$:	Voltage dependent calcium channel 1.2
$Ca_V1.3$:	Voltage dependent calcium channel 1.3
mRNA:	Messenger ribonucleic acid
ERK 1/2:	Extracellular signal-regulated kinases
SR:	Sarcoplasmic reticulum
TNFR2:	Tumor necrosis factor receptor 2
CD38:	Cluster of differentiation 38
cADPR:	Cyclic adenosine diphosphate ribose
p38 MAPKs:	p38 mitogen-activated protein kinases
JNK:	Jun NH_2-terminal kinase
MAPK:	Mitogen-activated protein kinases
$Ca_V1.1$:	Voltage dependent calcium channel 1.1
$Ca_V1.4$:	Voltage dependent calcium channel 1.4
i.p.:	Intraperitoneal
s.c.:	Subcutaneous
OA:	Ovalbumin
FBS:	Fetal bovine serum

Ser-NS: Serum from nonsensitized animals
SMI-TNF: Small-molecule inhibitor of TNF alpha
U0126: 1,4-Diamino-2,3-dicyano-1,4-bis-(0-amino-phenylmercapto)butadiene ethanolate, inhibitor of MEK
MT: Melting temperature
PBS: Sodium phosphate buffer
BP: Blocking peptide
EPI: Epithelium
SM: Smooth muscle
SOC: Store operated calcium channels
ROC: Receptor operated calcium channel
STIM: Stromal interaction molecule
SERCA: Sarcoplasmic reticulum calcium ATPase
NF-κB: Nuclear factor kappa B
AP-1: Activator protein
S^{496}: $Serine^{496}$
NIK: NF-kappaB-inducing kinase
IκKs: I kappa B kinases
MEK: Mitogen-activated protein kinase kinase
TRAF: TNF receptor-associated factor
IκBs: NF-kappa B inhibitors
RIP: Receptor interacting protein.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Jorge Reyes-García generated organ bath and patch clamp experiments and data analysis and contributed to writing of the paper. Edgar Flores-Soto and Héctor Solís-Chagoyán participated in organ bath and patch clamp experiments. Bettina Sommer contributed to the guinea pig sensitization process and writing of the paper. Verónica Díaz-Hernández and Luz María García-Hernández contributed to immunofluorescence experiments, mRNA extraction, and RT-PCR experiments. Luis M. Montaño contributed to the design and global supervision of the study, data analysis, and the writing of the paper.

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Review Article

Insulin Resistance and Endothelial Dysfunction Constitute a Common Therapeutic Target in Cardiometabolic Disorders

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Insulin resistance and other risk factors for atherosclerosis, such as hypertension and hypercholesterolemia, promote endothelial dysfunction and lead to development of metabolic syndrome which constitutes an introduction to cardiovascular disease. The insulin resistance and endothelial dysfunction cross talk between each other by numerous metabolic pathways. Hence, targeting one of these pathologies with pleiotropic treatment exerts beneficial effect on another one. Combined and expletive treatment of hypertension, lipid disorders, and insulin resistance with nonpharmacological interventions and conventional pharmacotherapy may inhibit the transformation of metabolic disturbances to fully developed cardiovascular disease. This paper summarises the common therapeutic targets for insulin resistance, endothelial dysfunction, and vascular inflammatory reaction at molecular level and analyses the potential pleiotropic effects of drugs used currently in management of cardiovascular disease, metabolic syndrome, and diabetes.

1. Introduction

Insulin plays an important role in maintenance of vascular homeostasis. On one hand insulin stimulates endothelial production of nitric oxide (NO), a crucial vasodilator exerting an antiaggregatory effect and limiting vascular smooth muscle cells growth and migration, but on the other one mediates the release of endothelin ET-1, known to act as a strong vasoconstrictor [1]. This dual action of insulin is mediated by two major signalling pathways. Under physiological conditions, a vasoprotective phosphoinositide-3-kinase (PI3-K)/Akt pathway predominates and is responsible for expression and activation of endothelial nitric oxide synthase (eNOS) [2].

When insulin resistance appears, the balance is shifted towards mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), which mediates inflammation, vasoconstriction, and vascular smooth muscle cell proliferation [3]. The crosstalk between insulin signalling pathways and endothelial metabolism is strongly related. Therefore, insulin resistance commonly coexists with endothelial dysfunction in cardiovascular disease. Both nonpharmacological and pharmacological interventions act on amelioration of insulin sensitivity as well as on improvement in endothelial function [4].

2. Insulin Signalling (Figure 1)

Insulin binds to insulin receptor IR, which contains the two α and two β subunits. The α subunit binds insulin, insulin growth factor-1 (IGF-1), and epidermal growth factor (EGF). The β subunit contains extracellular, transmembrane, and cytosolic domains. The cytosolic part of the β subunit has tyrosine kinase activity, which undergoes conformational changes and is autophosphorylated after insulin biding to the α subunit. Activated IR phosphorylates also number of proteins on tyrosine residues, for example, insulin receptor substrate (IRS), Shc proteins, or Gap-1 [5]. In human cells three isoforms of IRS (IRS-1, -2, and -4) were identified to play a distinct role, depending on cell type and metabolic

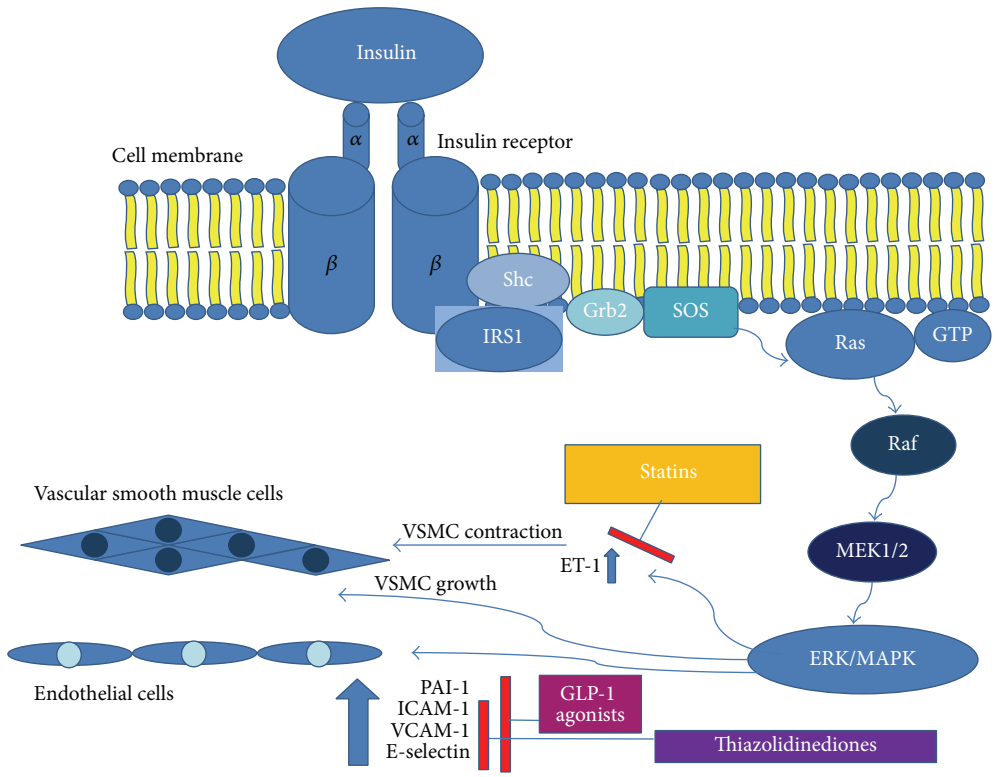

Figure 1: Insulin signalling in vessels. α: alfa subunit of insulin receptor; β: beta subunit of insulin receptor; Shc: Src homology and collagen protein; Grb2: cytosolic growth factor receptor-bound protein 2; IRS1: insulin receptor substrate; SOS: proline-rich domain of the son of sevenless; Ras: family of related proteins; Raf: serine/threonine specific protein kinases; MEK1/2: mitogen-activated protein kinase; ERK/MAPK: mitogen-activated protein kinase; ET-1: endothelin-1; GLP-1: glucagon-like peptide; PAI-1: plasminogen activator inhibitor; ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1.

state. Also those two insulin receptor substrates represent different kinetics, compartment distribution, and substrate interactions (IRS-1 is a transmembrane protein and IRS-2 is mostly present in cytosol) [6]. IRS-1 plays a crucial role in skeletal muscle and its function is to provide insulin secretion mechanisms [7]. IRS-2 is responsible for insulin action in liver and pancreatic β cells development. Animal models showed that IRS-1 knockout mice had growth retardation especially in skeletal muscle and liver, but not in brain [8]. Mice lacking IRS-1 developed insulin resistance with hyperinsulinemia, not diabetes, but displayed features of metabolic syndrome (hypertension and hypertriglyceridaemia) [8]. Animals without IRS-2 exhibited insulin resistance with fasting hyperglycemia, due to inadequate insulin production, which in final resulted in diabetes, which was worse than lack of IRS-1 [8]. IRS tyrosine phosphorylation is mandatory for insulin response, but depending on which serine is phosphorylated, IRS intensifies or diminishes insulin action [9].

3. The PI3-K/Akt Pathway

The phosphorylation of IRS tyrosine activates phosphoinositide-3 kinase (PI-3K), which converts phosphatidylinositol (3,4)-bisphosphate (PIP2) to a second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [10]. PIP3 facilitates translocation of Akt kinase from inactivated form to the cell membrane, where is activated by phosphoinositide-dependent kinase-1 (PDK-1) [11]. The Akt activation on Thr308 and Ser473 has many implications in cellular processes. Except for cell survival, growth, and proliferation, Akt influences also glucose metabolism, nitric oxide production, and angiogenesis [12]. In endothelial cells Akt activation may induce undesirable proliferation and survival of tumour vasculature [13], but in insulin resistant state diminished cell proliferation may lead to atherosclerosis, decreased collateral angiogenesis in occluded coronary and lower extremities vasculature, or reduced reendothelialisation [14]. The antiapoptotic effect of Akt phosphorylation is mediated by inhibition of caspase-9, which prevents endothelial cells from death induced by inflammatory response [15]. The crosstalk between endothelial cells and insulin signalling pathway is marked also in Akt phosphorylation at Ser1177 of endothelial nitric oxide synthase (eNOS) [2], which enhances antiapoptotic effect in ischemic myocardium and stimulates vasodilation and angiogenesis by nitric oxide production [16]. The eNOS activation is mediated by inhibition of calmodulin dissociation and electron transfer in a reductase domain [17]. Proangiogenic role of Akt is expressed by increased

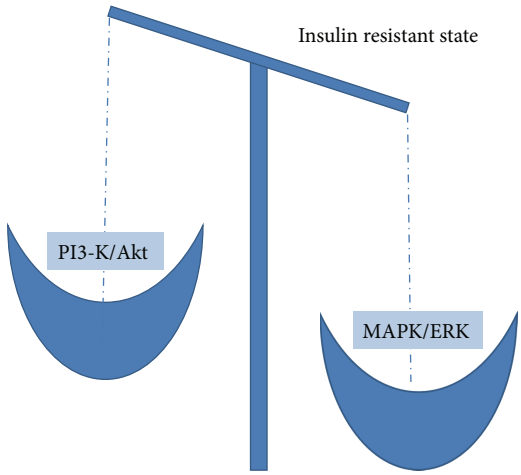

Figure 2: Conceptual definition of insulin resistance at molecular level.

production of the hypoxia-inducible factor α (HIF1α and HIF2α), which leads to secretion of proangiogenic factors for example vascular endothelial growth factor (VEGF) [18].

4. The MAPK/ERK Pathway

The MAPK pathway is activated by insulin, which results in cytosolic growth factor receptor-bound protein 2 (Grb2) binding to the plasma membrane. Grb2 interacts with IRS by Src homology and collagen protein (Shc). Grb2 is also associated with proline-rich domain of the son of sevenless (SOS), which is the guanyl nucleotide-exchange factor. This process triggers transformation of inactive GDP-bound Ras into active form of GTP-bound Ras [19]. Active Ras stimulates serine/threonine kinase Raf, which phosphorylates and activates MEK1/2. MEK1/2 phosphorylate in turn ERK, a member of the MAPK signalling enzymes [20]. MAPK pathway is also associated with endothelial cells by mediating secretion of ET-1 [21].

5. Insulin Resistance (Figures 2 and 3)

Insulin resistance refers to the state of decreased insulin response and is a common feature of obesity, hypertension, diabetes, and coronary artery disease [22]. Impairment of PI3-K/Akt signalling pathway leads to an inadequate tissue insulin sensitivity. The paradox of pathologies in molecular insulin signalling contributes to diminished activity of the PI3-K/Akt pathway coexisting with strengthened MAPK/ERK pathway, during compensatory hyperinsulinaemia [23]. Differences in activity of both pathways are responsible for divergences in insulin resistance in different organs for example lack of suppression of glucose production by insulin and maintained lipogenesis in the liver [24] or decreased production of nitric oxide and enhanced production of ET-1 in endothelium [25]. Insulin resistance is associated inseparably with glucotoxicity, lipotoxicity, and inflammation, which initiates and accelerates atherogenesis and vascular disease [26].

Changes in balance between the PI3-K/Akt and MAPK/ERK pathways provide strong relationship between insulin resistance and endothelial dysfunction [27]. What is more, when the balance in insulin resistance is shifted towards the MAPK/ERK pathway, it results in a release of inflammatory markers by insulin (e.g., PAI-1, ICAM-1, VCAM-1, and E-selectin) and finally promotes the endothelial dysfunction [28].

6. Endothelial Dysfunction (Figures 2 and 3)

Endothelium is a multifunctional paracrine, autocrine, and endocrine organ, "the ranger" of vascular homeostasis. The endothelial balance is maintained by substances of vasodilatory action (e.g., NO or prostaglandins (PGI2)) and vasoconstricting features (e.g., angiotensin II (Ang II) or ET-1) [29]. Insulin, by acting through distinct metabolic pathways, may influence both groups of factors. Activation of the PI3-K/Akt pathway leads to phosphorylation of eNOS and subsequent conversion of L-arginine to L-citrulline and NO, the most important vasodilator. NO plays also protective role for endothelium by decreasing expression of cell adhesion molecules, attenuating platelet aggregation, production of proinflammatory cytokines, and inhibiting vascular smooth muscle cells proliferation [30]. Deficiency in the NO bioavailability, increased level of prothrombotic and proinflammatory markers, and reactive oxygen species (ROS) are factors indicating endothelial dysfunction, which are mediated by MAPK/ERK activity. Glucotoxicity and lipotoxicity generate inflammatory reaction contributing to vascular damage and link insulin resistance with endothelial dysfunction through different mechanisms.

7. Glucotoxicity in Insulin Resistance and Endothelial Dysfunction

Hyperglycemia activates the hexosamine biosynthesis pathway and modifies proteins involved in insulin and NO signalling by the O-Glc-N-acylation of IRS-1, which impairs activation of PI3-K and reduces glucose uptake [31] and O-Glc-N-acylation of eNOS at the Akt phosphorylation residues, leading to its inactivation [32]. O-Glc-N-acylation also induces PAI-1 gene expression and alters tumor growth factor β (TGFβ) level, what is related to pathogenesis of vascular diabetic damage [33, 34]. The overactivation of hexosamine biosynthesis pathway results in formation of advanced glycation end products (AGEs), which in turn stimulate ROS production. Reactive carbonyl species (RCS) are formed in the course of oxidation of carbohydrates, lipids, and amino acids and have been identified as intermediates in the formation of irreversible, advanced glycoxidation and lipoxidation end products (AGEs and ALEs) on protein. Reactive carbonyl, oxygen, and nitrogen species (RCS, ROS, and RNS, resp.) are now recognized to be important transducers in biological systems. There is a growing body of population of structurally defined AGE products such as pyrraline, pentosidine, N-carboxy-methyl lysine (CML), and crossline that are found to be elevated in diabetic tissues.

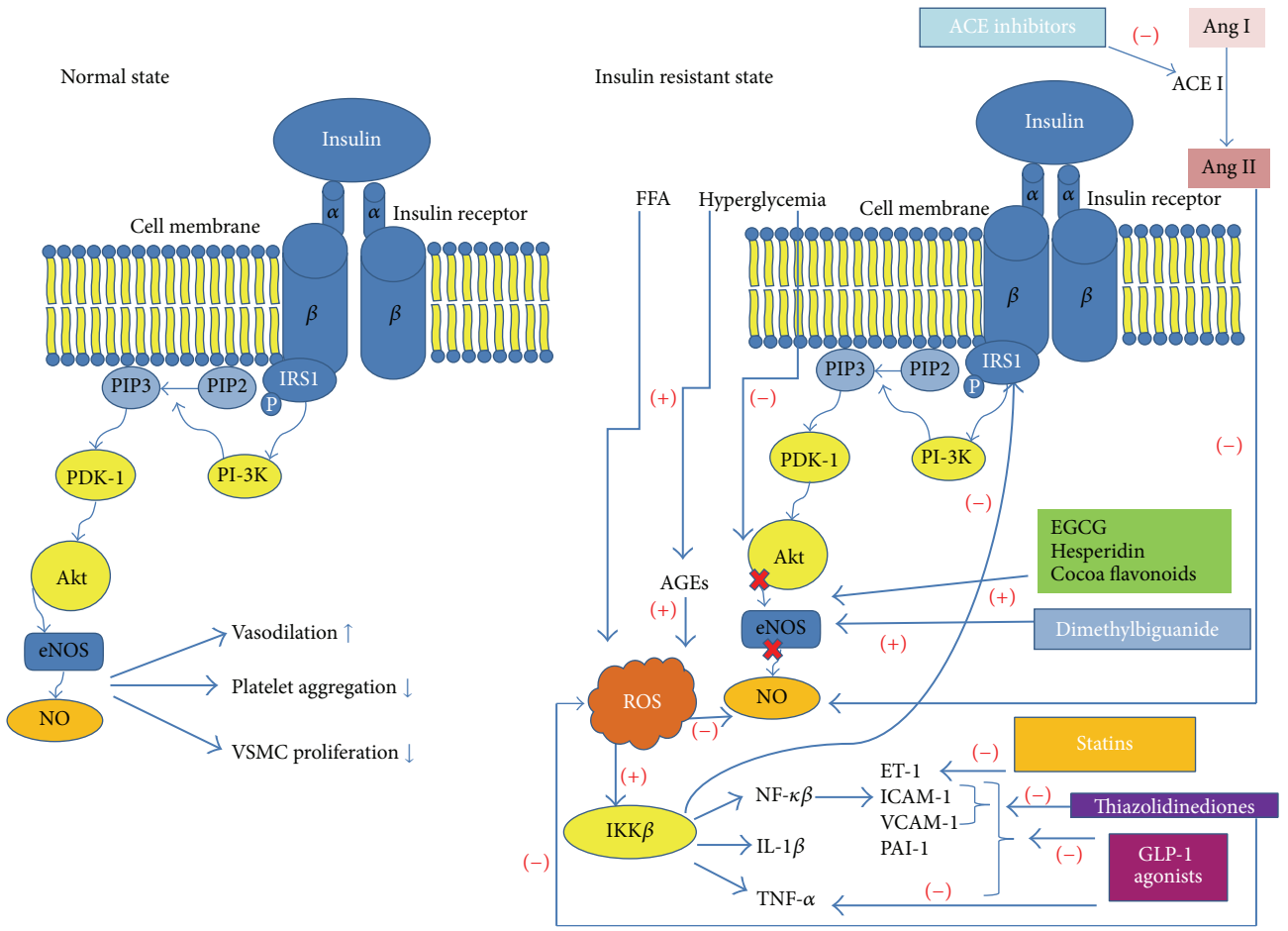

Figure 3: Changes in expression of particular molecular pathways in insulin resistant state versus under physiological condition. Molecular definition of drug targets for management of insulin resistance. α: alfa subunit of insulin receptor; β: beta subunit of insulin receptor; Shc: Src homology and collagen protein; Grb2: cytosolic growth factor receptor-bound protein 2; IRS1: insulin receptor substrate; SOS: proline-rich domain of the son of sevenless; Ras: family of related proteins; Raf: serine/threonine specific protein kinases; MEK1/2: mitogen-activated protein kinase; ERK/MAPK: mitogen-activated protein kinase; ET-1: endothelin-1; GLP-1: glucagon-like peptide; PAI-1: plasminogen activator inhibitor; ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1; Ang I: angiotensin I; Ang II: angiotensin II; ACE I: angiotensin converting enzyme I; TNF-alpha: tumour necrosis factor alpha; ROS: reactive oxygen species; Akt: the Akt kinase; eNOS: endothelial nitric oxide synthase; AGEs: advanced glycation end products.

Some of the highest levels of pentosidine have been detected in individuals with diabetes. There is also some evidence for elevated skin pentosidine levels in individuals with diabetes correlate with the severity of the complications [35–37].

Increased oxidative stress enhances insulin resistance by impairing Akt and eNOS activation and limiting NO availability [38]. Moreover, ROS stimulates IKKβ kinase, which leads to activation of NF-κB and overexpression of proinflammatory markers, for example, interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), and phosphorylation and inactivation of IRS-1 [39]. ROS forming oxidant peroxynitrites ($ONOO^-$) enhance endothelial dysfunction by direct uncoupling and inactivating the eNOS.

Modification of endothelial cells matrix collagen and laminin by AGEs impairs vascular elasticity and interaction with macrophages promotes atherosclerosis [40]. Vascular remodelling of vessels associated with cardiometabolic disorders seems to be hypertrophic and it is mostly due to increased extracellular matrix deposition. The mechanisms underlying the obesity-, insulin resistance-, and/or hyperinsulinemia-induced vascular disease are not fully understood but might include hemodynamic factors such as hypertension, activation of the renin-angiotensin-aldosterone system, metabolic factors such as insulin and advanced glycation end products, and other factors such as adipokines, inflammation, or oxidative stress [41]. Hyperglycemia promotes AGEs production, which inhibit tyrosine phosphorylation of IRS-1 and IRS-2 and decrease activation of the PI3-K/Akt pathway by activation of phosphokinase C (PKC) [42]. Adipose tissue has been demonstrated to be an active organ, where matrix metalloproteinases (MMPs) play an important role in adipogenesis, angiogenesis, and proliferation of extracellular matrix. However, the lack of association between adipose tissue and plasma levels of some MMPs, specifically MMP-2

and MMP-9, suggests that this tissue is not a major contributor to circulating MMPs. These enzymes, which are responsible for tissue remodelling, are also expressed in response to inflammatory adipocytokines, like adiponectin or leptin. Adiponectin may also play a protective role in the plaque rupture through selectively increasing the tissue inhibitor of metalloproteinase (TIMP) expression. Leptin induces expression of MMP-2 activators and the expression of MMP-2, MMP-9, and TIMP-1 in numerous human cells [43].

8. Lipotoxicity in Insulin Resistance and Endothelial Disfunction

Lipotoxicity inhibits the PI3-K/Akt signalling and activates the MAPK/ERK pathway by inducing oxidative stress and inflammation through free fatty acids (FFA) [44]. FFA stimulate PKC impairing Akt function due to IRS 1/2 inactivation [45] and enhance NADPH oxidase to ROS production [46]. NADPH oxidase induces production of PAI-1, interleukin-6 (IL-6), and chemokine (C-C motif) ligand 2 (CCL-2), which increase proinflammatory state and inhibits NO production by decreasing eNOS expression. Moreover, ROS after FFA stimulation activate NF-κB, which increases ET-1 expression and adhesion molecules ICAM-1 and VCAM-1 and increase cardiovascular risk even in healthy subjects [47].

The adhesion molecules on endothelial cells promote their contact with monocytes, which turn into macrophages absorbing lipoproteins and as the foam cells secrete IL-6 and TNF-α. TNF-α and IL-6 mobilize immune cells to build atherosclerotic plaque and activate IKKβ, which leads to impaired insulin signalling in endothelial cells and activates NF-κB [48].

9. Nonpharmacological Interventions Improving Insulin Sensitivity and Endothelial Function

An imbalance between the PI3-K/Akt and MAPK/ERK pathways links insulin resistance and endothelial dysfunction. Pathology of decreased activation of Akt signalling with diminished NO production and stimulation of MAPK pathway is commonly contributed to overweight, obesity, and diabetes [49]. Dietary intervention leading to restoration of the balance between both pathways targets insulin sensitivity and endothelial function. There are animal and human studies demonstrating beneficial effect of polyphenols added to diet, based on their involvement in pathways described above. Green tea polyphenol (EGCG) has been discovered to mimic insulin action via PI3-K pathway, by stimulating glucose uptake and inhibiting hepatocyte gluconeogenesis [50]. Moreover, EGCG is involved in pathway regulating eNOS activation and NO production in endothelium [51]. This feature of green tea polyphenol contributes to its antidiabetic, insulin sensitizing, and lipid lowering properties [52]. Another floral eatable polyphenol of eNOS activating effect is hesperidin, extracted from citrus fruit. Hesperidin is shown to reduce the triacilgliceroles level and free fatty acid oxidation with decrease in inflammatory markers [53]. Cocoa flavonoids also showed positive effect on eNOS activity and endothelial improvement as well as on insulin sensitivity in several short-term studies [54]. Animal studies involving dietary restriction of AGEs elimination also revealed satisfying effect on metabolic disturbances. Reversing insulin resistance combined with suppressing the inflammation and atherosclerosis might be a future therapeutic option [55]. AGEs are absorbed from highly heated processed food (barbeque, grilled) and higher levels were shown to correspond with vascular damage [56]. However, low-quality evidence of human studies needs further investigation [57].

Meta-analyses, which compared different dietary patterns, have shown that the Mediterranean diet has beneficial effect on cardiovascular disorders, cancer risk [58], and diabetes [59]. Nonpharmacological interventions combine also diet with physical exercise, which is demonstrated to reduce inflammatory markers and improve insulin sensitivity [60]. Lifestyle modifications can stop and reverse disease, which was shown by Esposito et al. by comparing Mediterranean to low-fat diet with an effect of remission of diabetes and delay of drug requirement [61].

10. Pharmacological Interventions Improving Insulin Sensitivity and Endothelial Function (Figure 2)

10.1. Thiazolidinediones. Thiazolidinediones bind to peroxisome proliferator-activated receptor (PPAR-γ). PPAR-γ regulates transcription of insulin sensitive genes, which control glucose and lipid metabolism. Thiazolidinediones improve insulin sensitivity and decrease FFA circulating amounts [62]. Their anti-inflammatory properties are expressed by decrease in expression of adhesion molecules, ICAM-1, VCAM-1, and E-selectin, which protect monocytes from vascular wall attachment and later lipid accumulation in macrophages [63]. PPAR-γ ligands inhibit NFκB and decrease inflammation that way. Thiazolidinediones inhibit NADPH oxidase expression components NOX1, NOX2, and NOX4, reduce ROS production, increase NO formation through heat shock protein 90 and eNOS interaction [64], promoting vasodilation, and suppress ET-1, protecting from vasoconstriction. PPAR-γ ligands decrease vasculature complications in diabetes, by lowering fasting insulin level and blood pressure, and reduce secondary clinical end point of stroke and myocardial infarction death.

10.2. Dimethylbiguanide. Metformin is an oral first-line treatment in diabetes 2 and is not associated with a hypoglycemic tendency. Metformin exerts its antihyperglycemic effect by decreasing hepatic glucose production by suppressing of gluconeogenesis and enhancing insulin suppression of endogenous glucose production, by reducing intestinal glucose reabsorption and possibly improving glucose uptake and utilization by peripheral tissues, such as skeletal muscle, and adipose tissue lowers blood glucose levels [65]. It acts via the AMP-activated protein kinase (AMPK) and by eNOS phosphorylation and NO increased production

ameliorates endothelial function [66]. Despite AMPK pathway, metformin inhibits the respiratory chain complex-1 (NADH:ubiquinone oxidoreductase) in mitochondria [67] and regulates oxidative stress induced by hyperglycemia. Metformin plays also a crucial role in the incretin pathway through the glucagon-like peptide (GLP-1), by enhancing its production [68].

10.3. Glucagon-Like Peptide-1 Connected Drugs. GLP-1 is a hormone generated and secreted from enteroendocrine cells of intestine, which enhances glucose-stimulated insulin secretion and suppresses glucagon release thereby modulating both postprandial and long-term glucose homeostasis [69]. It acts through the G-protein coupled receptor (GLP-1R). GLP-1 is inactivated by the serine protease dipeptidyl-peptidase-4 (DPP-4) [70]. Soluble form of dipeptidyl-peptidase-4, which is present in plasma, is inactive against novel diabetic drugs degradation-insensitive GLP-1R agonists (exenatide, liraglutide, and lixisenatide). Liraglutide (NN2211) is a long-acting glucagon-like peptide-1 receptor agonist, binding to the same receptors as does the endogenous metabolic hormone GLP-1 that stimulates insulin secretion. Exenatide (NN2211) is a 39-amino acid peptide, an insulin secretagogue, with glucoregulatory effects, and is a long-acting glucagon-like peptide-1 receptor agonist, binding to the same receptors as does the endogenous metabolic hormone GLP-1 that stimulates insulin secretion. Lixisenatide has been described as "des-38-proline-exendin-4 (*Heloderma suspectum*)-(1–39)-peptidylpenta-L-lysyl-L-lysinamide," meaning it is derived from the first 39 amino acids in the sequence of the peptide exendin-4, found in the Gila monster (*Heloderma suspectum*), omitting proline at position 38 and adding six lysine residues [71].

GLP-1 has vasoprotective properties, including its effects on heart rate, ischemia/reperfusion injury, coagulation, inflammation, and vascular endothelial function [72]. GLP-1 agonists reveal vasodilatory properties, by increasing the NO production, stimulating proliferation, and protecting from lipid-induced apoptosis of human endothelial cells, through PI3K/Akt pathway, protein kinase A (PKA), and the eNOS-dependent pathways [73]. Liraglutide reduces inflammatory cytokine (TNF-α) and hyperglycemia-induced expression of the fibrinolysis inhibitor, PAI-1, and vascular adhesion molecules VCAM-1 and ICAM-1, which decreases inflammation and monocytes attachment [74]. In animal models GLP-1 agonist diminished monocyte adhesion, macrophage infiltration, and atherosclerotic lesions in the vasculature [75].

High activity of the DPP-4 enzyme in immune system might give a possibility of using dipeptidyl peptidase-4 inhibitors in anti-inflammatory therapy, particularly in atherosclerosis. DPP-4 inhibitors mediate macrophages polarization in atherosclerotic regions, decrease the level of M1 macrophages, responsible for inflammation [76], and expand anti-inflammatory M2 macrophages, which, in turn, might diminish insulin resistance and ameliorate endothelial function. Inflammatory reactions might be reduced by GLP-1R agonists and DPP-4 inhibitors, due to macrophages shift into M2 type through T regulatory lymphocytes (Tregs), whose function is increased by GLP-1 [77]. Moreover, Tregs secrete interleukin-10 (IL-10), which inhibits NADPH oxidase, reducing oxidative stress and ROS production. This additional metabolic role protects endothelium and maintains correct insulin signalling, since NADPH oxidase has been shown to activate serine kinases, which phosphorylate IRS and disrupt physiological insulin pathway [78]. Pharmacological inhibition of dipeptidyl peptidase-4 increase the bioavailability of GLP-1, which enhances insulin-dependent action in vasculature. Saxagliptin (rINN), previously identified as BMS-477118, linagliptin (BI-1356), vildagliptin (LAF237), sitagliptin (MK-0431), and alogliptin are oral hypoglycemic agent of the dipeptidyl peptidase-4 (DPP-4) inhibitor class of drugs approved by the FDA for management of type 2 diabetes in adults. Animal studies of obese Zucker rats treated with linagliptin showed improvement in eNOS activation, blood pressure, and diastolic heart function [79]. Nonetheless, two large clinical studies with DPP-4 inhibitors, EXAMINE [80], which involved alogliptin, and SAVOR-TIMI 53 [81], which involved saxagliptin, did not show reduced risk of cardiovascular events, but further investigations are needed [82].

11. Drugs Acting on the Renin-Angiotensin-Aldosterone System

In insulin resistance and endothelial dysfunction, a hyperactivity of the renin-angiotensin-aldosterone system (RAAS) plays a crucial role, and therefore targeting it on a different molecular level benefits in improvement in insulin sensitivity and vascular function. The most harmful factor in this system, affecting insulin metabolism and endothelium, is angiotensin II (Ang II). Ang II is converted from inactive angiotensin I by the angiotensin converting enzyme (ACE) and acts as a ligand for angiotensin II receptors, mostly type 1 (AT1). Angiotensin II interferes with the insulin pathways by suppressing IRS-1 phosphorylation and decreasing PI3-K function and glucose receptor (GLUT-4) translocation, which diminishes glucose uptake [83]. Moreover Ang II hinders endothelial function by decreasing NO bioavailability through NADPH oxidase activation and ROS production. Destructive function of Ang II affects also endothelium by enhancing NF-κB, which in turn promotes production of TNFα and IL-6 and adhesion molecule VCAM-1, mediating inflammation [84]. Association between RAAS, insulin, and endothelial pathways results in wide use of drugs targeting those common pathologies, and therefore the treatment with ACE inhibitors, which reduce circulating AngII levels and angiotensin receptor blockers (ARBs), has additional benefits beyond antihypertensive effect. This metabolic outcome results from blocking the crosstalk between Ang II and insulin at the level of IRS-1 and PI3-K [85]. Human and animal studies showed that ACE inhibitors and ARBs have positive effect on glucose disposal in glucose intolerance, diabetes mellitus, obesity, and hypertension [86]. In line with these reports, some trials have shown that ACE inhibitors and ARBs improve insulin sensitivity and prevent new onset of diabetes [87]. In the DREAM trial (Diabetes Reduction

Assessment with Ramipril and Rosiglitazone Medication) ramipril reduced the postchallenge glucose levels and increased the tendency of regression to normoglycemia in subjects with impaired glucose tolerance and impaired plasma glucose levels [88]. In the TREND study (Trial on Reversing Endothelial Dysfunction) another ACE inhibitor, quinapril, has been shown to improve endothelial function by enhancement in the NO release in normotensive subjects with coronary artery disease [89]. Increase in NO production might facilitate glucose delivery to tissues due to vasodilation. ARB representative, losartan, also increased insulin sensitivity, improved endothelial function, and impacted inflammatory markers in hypercholesterolemic hypertensive patients [90]. Different mechanisms of crosstalk between insulin and endothelial pathways are perfectly optimized during telmisartan treatment, due to its dual action, which consists of angiotensin receptor blockade and activation of the peroxisome proliferator-activated receptor-γ (PPAR-gamma) [91].

12. Hypolipemic Drugs

In pathologies accompanied by hyperlipidemia, the two types of therapeutic regimens are commonly used: the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) and fibrates. Statins are characterized by improving endothelial function, reducing inflammation and ET-1 circulating levels, which diminishes vasoconstriction and ameliorates the insulin activity [92], especially in addition to ACE inhibitors or ARBs. Fibrates act as a PPAR-α ligands improving lipid profile, insulin sensitivity, and endothelial function and diminishing vascular inflammation, which has been shown in the FIELD study [93].

13. Conclusions

Endothelial and insulin signalling pathways crosstalk each other and therefore the relationship between endothelial function and insulin metabolism is very important in disorders, such as hypertension, obesity, or diabetes. Insulin resistance, a hallmark of metabolic syndrome, impairs vascular response and increases cardiovascular risk. Involvement of insulin resistance and endothelial dysfunction in pathological disorders contribute to impairment in the NO-dependent vasodilatation, cellular glucose uptake, enhancement in oxidative stress, and inflammation, leading finally to atherosclerosis. Strong association of insulin and endothelial signalling disturbances contributes to glucotoxicity, lipotoxicity, and inflammation, disrupting the balance between vasodilating-vasoconstrictive endothelial mechanisms as well as between the insulin-dependent PI3-K/Akt–MAPK/ERK pathways. The synergistic antidiabetic, antihypertensive, and hypolipemizing treatment, aiming at multiple metabolic pathways, improve both insulin sensitivity and endothelial function and should be considered at early stages of disturbances, before clinical progression of diseases, with fully developed vascular complications.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Role of *Porphyromonas gingivalis* HmuY in Immunopathogenesis of Chronic Periodontitis

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Periodontitis is a multifactorial disease, with participation of bacterial, environmental, and host factors. It results from synergistic and dysbiotic multispecies microorganisms, critical "keystone pathogens," affecting the whole bacterial community. The purpose of this study was to review the role of *Porphyromonas gingivalis* in the immunopathogenesis of chronic periodontitis, with special attention paid to HmuY. The host response during periodontitis involves the innate and adaptive immune system, leading to chronic inflammation and progressive destruction of tooth-supporting tissues. In this proinflammatory process, the ability of *P. gingivalis* to evade the host immune response and access nutrients in the microenvironment is directly related to its survival, proliferation, and infection. Furthermore, heme is an essential nutrient for development of these bacteria, and HmuY is responsible for its capture from host heme-binding proteins. The inflammatory potential of *P. gingivalis* HmuY has been shown, including induction of high levels of proinflammatory cytokines and CCL2, decreased levels of IL-8, and increased levels of anti-HmuY IgG and IgG1 antibodies in individuals with chronic periodontitis. Therefore, the HmuY protein might be a promising target for therapeutic strategies and for development of diagnostic methods in chronic periodontitis, especially in the case of patients with chronic periodontitis not responding to treatment, monitoring, and maintenance therapy.

1. Introduction

Periodontal diseases are among the most common chronic inflammatory diseases in humans [1]. They comprise a number of inflammatory and infectious conditions caused by the inflammatory host response to bacteria in the supragingival and subgingival biofilm. The presence of periodontal pathogens may lead to an imbalance in the periodontal environment, and the subsequent host innate and adaptive immune response may lead to soft and/or hard tissue destruction. Periodontal pathogens composing a biofilm can injure periodontal tissues by way of the inflammatory response. Periodontitis may affect the gingiva, causing gingivitis, or may progress to the supporting periodontium, potentially affecting tooth mobility, which may lead to tooth loss [2]. Localized and aggressive forms of periodontitis are associated with *Aggregatibacter actinomycetemcomitans*, while chronic forms of generalized disease involve other bacteria, including *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, and *Treponema denticola* [3]. Periodontal diseases are modulated by the immune response and can be a risk factor for systemic disorders. Current evidence supports the importance of several factors increasing onset and progression of periodontal diseases, including smoking [4]. Tobacco use can also lead to diabetes mellitus, which may influence inflammatory changes in periodontal tissues. Other potential interactions with periodontal disease are still being investigated, such as those involving obesity, hormonal changes, cardiovascular and respiratory diseases, and adverse pregnancy outcomes [5, 6].

Several recent studies have proposed a new model of pathogenesis for periodontitis, pointing to a synergistic and dysbiotic microbial community responsible for the initiation of periodontal diseases, instead of the action of selected periodontal pathogens [7–9]. Bacteria termed "keystone pathogens," found in low abundance under healthy conditions, can destabilize the community and cause the development of dysbiosis. The best-documented example of such pathogens is *P. gingivalis*, an anaerobic Gram-negative coccobacillus which belongs to the Bacteroidaceae family. In the natural environment, *P. gingivalis* is a constituent of the multispecies biofilm [10, 11]. The bacterium can also enter gingival epithelial and immune cells, remain viable and capable of spreading among cells [12–14], and spread systemically to other tissues [15–19]. A number of studies have demonstrated that *P. gingivalis* is localized in various subcellular compartments of host cells, including cytoplasm, endosomes, and autophagosomes. It has been found that the bacterium instead of trafficking to the endosomal pathway traffics to the autophagosome-like vacuoles and resides in vacuoles that resemble early and late autophagosomes, which may allow survival by blocking fusion with lysosomes [12, 20, 21]. Bacterial trafficking to the autophagic pathway allows protection from the host's defense mechanisms and acquisition of nutrients, which is especially beneficial for asaccharolytic *P. gingivalis*. Moreover, outer-membrane vesicles produced by *P. gingivalis* enter human cells via a lipid raft-dependent endocytic pathway, are routed to endosomes, and are sorted to lysosomal compartments [22, 23]. All these data suggest that this pathogen has the ability to invade host cells [24], which can be an escape mechanism from host defenses, favoring the microorganism's penetration in the bloodstream and thus acting systemically in the host body [25].

Important features of *P. gingivalis*-mediated chronic periodontitis include the ability of the bacterium to adhere to and invade host cells, disseminate through host cells and tissues, and subvert host immunological surveillance and defense mechanisms. However, virulence determinants of periodontopathogens that allow for efficient infectivity and promote synergy in the increase of virulence are still not clear. The aim of this review is to present the role that *P. gingivalis* antigenic determinants play in the immunopathogenesis of chronic periodontitis, with special attention paid to the *P. gingivalis* HmuY protein.

2. Immunopathogenesis of Chronic Periodontitis

While bacterial infection is the primary etiologic factor, it is not sufficient to induce the onset and progression of periodontitis. A localized inflammatory reaction is stimulated by bacteria components, resulting in activation of the host innate immune system. The innate response involves the recognition of microbial components by Toll-like receptors (TLRs) expressed by host cells in the infected microenvironment [26]. Activation of these cells leads to the release of proinflammatory cytokines and the recruitment of phagocytes and lymphocytes. The activation of T lymphocytes initiates an adaptive immune response, Th1, Th2, Treg, or Th17, whereas B lymphocytes also participate in this process via the production of antibodies [27].

$CD4^+$ and $CD8^+$ T cells become activated following the recognition of microbial components, and many functionally distinct subsets of these lymphocytes have been described, each expressing different cytokines and transcription factors. NF-kappaB (NFκB) is a key transcription factor complex that appears to play a critical role in the regulation of an acute inflammatory response by activating a cascade of cytokines and producing other proinflammatory mediators, including adhesion molecules (e.g., ICAM-1, VCAM-1, and E-selectin), enzymes (e.g., COX-2, 5-LO, CPLA, and iNOS), cytokines (e.g., IL-1, TNF, IL-6, GM, and G-CSF), and chemokines (e.g., IL-8, RANTES, MCP-1, eotaxin, and MIP-1κ) [28–30]. The activation of NFκB may be inhibited by NFKBIL1 expression [31]. Genetic variations in NFKBIL1 are associated with susceptibility to inflammatory and/or autoimmune diseases [32].

Activated naive $CD4^+$ T cells may differentiate into either Th1 lymphocytes expressing T-bet transcription factor, IL-2, IFN-γ, and TNF-β, or Th2 lymphocytes, which express GATA-3 and IL-4, IL-5, and IL-13, or Th17 or RORγt lymphocytes expressing IL-17A, IL-17F, IL-21, and IL-22. The specific cytokines produced play a role in determining the inflammatory process. Recent analyses of cytokine profiles and transcription factors have shown that Th17, Tf-h, Th9, and Th22 profiles may become activated in periodontal diseases [33–38]. Effector T cells may become naive, recently activated, or become memory T cells that can be distinguished by cell surface markers [39].

Recent studies have demonstrated the involvement of IL-33 in the pathogenesis of periodontitis [40–42]. This cytokine has been described as an IL-1 family member that is expressed by many cell types following proinflammatory stimulation and participates in cell lysis mechanisms. Increased levels of IL-33 secreted in periodontal tissues can exacerbate the periodontal destruction induced by RANKL [40, 43]. Some studies have shown a positive association between elevated levels of IL-33 in periodontal tissue and periodontitis [44, 45]. However, this issue is still controversial and some authors have suggested that this cytokine plays a protective role by inducing a predominant Th2 profile [42]. Several studies have shown that also IL-18 can influence the pathogenesis of chronic periodontitis [46–51]. IL-18 is a potent proinflammatory cytokine with structural similarity to IL-1β [50]. In the presence of IL-12, IL-18 induces a Th1 response, whereas, in the absence of IL-12, a Th2 response is promoted [52]. CD4+ T cells also secrete proresorptive cytokines, such as IL-1, IL-6, and IL-17, and each of these cytokines stimulates the expression of the NFκB ligand (RANKL) receptor activator in osteoblasts and fibroblasts, which promotes osteoclast formation via a contact-dependent process [53]. IL-10 may also be present in the microenvironment of periodontal lesions, promoting negative feedback by various cell types, including T cells, B cells, macrophages, NK cells, mast cells, and neutrophils. Additional negative effects of IL-10 include the modulation of IL-1, IL-8, IL-12, and TNF-α and the inhibition of phagocytosis [54].

Typically, the T cell repertoire contains $CD4^+$ $CD25^+$ T regulatory lymphocytes that control the autoreactive

peripheral immune response [54]. The populations of $CD4^+$ $CD25^+$ T regulatory cells in periodontal disease have been shown to be higher in periodontitis compared to gingivitis [55]. T regulatory cells are responsible for mechanisms of tolerance, and the suppressive function of $CD4^+$ $CD25^+$ cells was found to be partly dependent on cell contact, suggesting that the human mucosa induces tolerance to different antigens [56]. T regulatory cells (Treg) [57, 58] and Th17 cells [59, 60] have been identified in periodontal tissues, suggesting the importance of immunoregulation in periodontal diseases. The clinical implications of these studies can be seen in the identification of Th1/Th2 and Treg/Th17 genes in peripheral blood and salivary transcriptomes. The recognition of their role in immunopathogenesis of periodontitis is being tested to evaluate their potential as markers of susceptibility for this disease [61].

3. Antigenic Determinants of *Porphyromonas gingivalis*

P. gingivalis strains 33277, 381, and A7436 can locally invade periodontal tissues and evade host defense mechanisms, using a range of virulence factors that disrupt the innate immune and inflammatory responses [62]. A variety of virulence factors of this bacterium, such as capsule components, lipopolysaccharides (LPS), fimbriae, proteases, and outer-membrane proteins, can promote immunogenicity by stimulating a mechanism of innate and adaptive immunity, in both the host humoral immune response and the host cellular immune response.

3.1. Gingipains. Among a variety of secreted and structural components that contribute to *P. gingivalis* virulence are arginine-specific gingipains (HRgpA and RgpB) and lysine-specific gingipain (Kgp) [8, 9, 12, 63]. Many studies have demonstrated that the protease activity of gingipains is responsible for a variety of virulent features of *P. gingivalis* and survival of this pathogen in host cells. However, contradictory roles of gingipains in the manipulation of host defense systems by *P. gingivalis* have been reported, since they act by both stimulating and inhibiting innate immune responses [64]. Moreover, *P. gingivalis* (strain HG66) HRgpA and Kgp, but not RgpB, mediate in a proteolytic-independent manner enhancement of production of proinflammatory cytokines in macrophages [65]. Such an effect may be caused by hemagglutinin/adhesion domains of Kgp and HRgpA. Gingipains produced by *P. gingivalis* participate in several mechanisms of host protein activation and deactivation by stimulating the expression of matrix metalloproteinases (MMPs) in fibroblasts [66–70]. MMPs are a group of zinc-dependent enzymes responsible for the degradation of the extracellular matrix during tissue renewal and also during inflammatory processes. They normally exhibit low levels of expression and activity in adult tissues but may be significantly increased in a variety of pathological conditions, thereby leading to tissue destruction by way of inflammatory disorders, tumor growth, and metastasis. Gingipains can cleave and degrade collagen and the connective tissue matrix under normal physiological pH and temperature conditions. When secreted, they can destroy periodontal tissues, degrade cytokines, deactivate the host's complement system components, and cleave various receptors, including CD14 on macrophages and CD4 and CD8 on T cells, thereby inhibiting host defense systems and facilitating *P. gingivalis* colonization [67].

3.2. LPS. The LPS of *P. gingivalis* is structurally different from the LPS of other Gram-negative bacteria and also has different immunogenic properties. It is recognized in innate host cells by TLR-2 [71–73] and can interact with TLR-2 and TLR-6 [73]. This unusual recognition pattern depends on the structural heterogeneity of lipid A [74], which enables connection to both TLR-2 and TLR-4 in association with CD14. Furthermore, a lipoprotein associated with LPS of *P. gingivalis* 381 strain (encoded in W83 under the PG1828 locus) appears to be involved in signaling through TLR-2, and its removal can markedly reduce the recognition of *P. gingivalis* strains 33277, A7436, 381, and W50 [75].

3.3. Other Proteins. Recently, an important role in immune signaling pathways was ascribed not only to *P. gingivalis* gingipains and LPS, but also to other proteins produced by this bacterium, including serine phosphatase (SerB), peptidyl arginine deaminase, nucleoside diphosphate kinase, and fimbriae (FIMA and MFA1), FimA, HemB, HbR, Hgp44, and RagB [76–78]. It has been shown that FimA signals through TLR2 and TLR4, and HemB signals through TLR4 [25, 74, 79, 80].

3.4. HmuY and Heme Uptake. The capacity of *P. gingivalis* to evade the host immune response and obtain nutrients in the microenvironment is directly related to its survival, proliferation, and infection, and one of the essential nutrients in the host environment is heme. To acquire heme as a main source of iron and protoporphyrin IX (PPIX), *P. gingivalis* strains A7436 and W83 have evolved sophisticated mechanisms that enable uptake of this compound bound to host hemoproteins [81–83]. To acquire heme, *P. gingivalis* uses hemagglutinins, proteases (particularly gingipains), lipoproteins, and outer-membrane receptors [67, 84–86]. It has been shown that Kgp and HRgpA can bind and subsequently cleave hemoglobin [87]. HmuR, an outer-membrane TonB-dependent receptor, is involved in heme transport through the *P. gingivalis* A7436, W83, and 381 outer membrane [88, 89], whereas HmuY is a membrane-associated heme-binding protein [90–92]. These proteins serve as the first example of a novel, two-component system, comprising an outer-membrane receptor and heme-binding hemophore-like protein.

The HmuY protein is associated with the outer membrane of the bacterial cell and outer-membrane vesicles through the lipid anchor [91, 92] and can be shed in an intact, soluble form through the limited proteolytic activity of *P. gingivalis* strains' (A7436, W83, and ATCC 33277) Kgp [86, 91]. HmuY may be functional in the form of dimers or tetramers. Both HmuY and HmuR appear to be essential for survival and growth of *P. gingivalis*, since *hmuY* and *hmuY-hmuR* mutant strains constructed in the A7436 strain showed defects in growth when heme and hemoglobin were used as the sole iron and PPIX sources [89]. Moreover, recent data demonstrated that

HmuY is required for effective *in vivo P. gingivalis* growth and invasion of macrophages [93]. It has been shown that the HmuY protein is resistant to several proteases, including *P. gingivalis* gingipains and *P. intermedia* interpain A (InpA) [86, 94], as well as host proteases, including neutrophil elastase [95], which allows its distribution and persistence in the host environment.

3.5. Role of HmuY Protein in Cooperation/Competition with Other Periodontopathogens. Recent studies have shown syntrophy between different bacterial species within oral biofilm through mutual cooperation/competition for nutrient acquisition, especially between *P. gingivalis*, *T. denticola*, *P. intermedia*, and *T. forsythia*, which form a polymicrobial community and dominate the periodontal biofilm [96]. Recently, it has been shown that black pigmented anaerobes, including *P. gingivalis*, display a novel heme acquisition paradigm, whereby hemoglobin must be first oxidized to methemoglobin, facilitating heme release [97]. In the case of *P. gingivalis* strains W83 and W50, this involves the arginine-specific gingipain proteases [98] and in the case of *P. intermedia* the protease InpA [94, 99]. The bacteria are then able to fully proteolyze the more susceptible methemoglobin substrate to release free heme. Importantly, HmuY is resistant to proteolysis and thus able to cooperate with *P. gingivalis* gingipains or *P. intermedia* InpA to extract the heme from hemoglobin, previously converted proteolytically into methemoglobin.

3.6. HmuY and the Host Immune Response. Several reports have demonstrated that not only do *P. gingivalis* cells localize in various cellular compartments of host cells, including cytoplasm, endosomes, and autophagosomes [12, 20, 21, 100], but also outer-membrane vesicles produced by *P. gingivalis* enter human cells via a lipid raft-dependent endocytic pathway, are routed to endosomes, and then are sorted to lysosomal compartments [22, 23, 101]. After lysis of *P. gingivalis* cells or outer-membrane vesicles, their antigens can be identified and processed by antigen-presenting cells, such as macrophages, causing activation of the adaptive immune response and production of antibodies. We recently demonstrated that also HmuY protein, both the protein produced by live *P. gingivalis* cells and the soluble protein added to macrophage cultures either together with the *hmuY* mutant strain constructed in the A7436 strain or alone, is internalized in macrophages [93]. Therefore, the HmuY protein can be recognized by the host immune system during chronic periodontitis. Indeed, we found that anti-HmuY antibodies inhibited *P. gingivalis* growth and biofilm formation [91]. Antibodies directed against *P. gingivalis* A7436 and ATCC 33277 HmuY are highly specific for the purified HmuY protein and cell-bound HmuY and do not cross-react with HmuY homologs found in *P. intermedia* and *T. forsythia* [102]. This suggests that *P. gingivalis* ATCC 33277, W83, and A7436 HmuY can serve as a specific antigen for serum determination of antibodies raised against this bacterium in patients with chronic periodontitis [103].

The immunogenic potential of *P. gingivalis* HmuY has been further demonstrated through promotion of inflammation, mainly by inducing high levels of IL-1β and IL-6 [103, 104]. Furthermore, HmuY seems to participate in a delayed host response through the increase of IL-10 and IL-6 levels and IgG and IgG1 levels of anti-HmuY antibodies and decrease of IL-8 levels in individuals with chronic periodontitis [104, 105]. *P. gingivalis* HmuY also evokes inflammatory responses in peripheral blood mononuclear cells (PBMC) derived from chronic periodontitis individuals, eliciting CCL2 and IL-18 as well as inhibiting NFKBIL-1 and IL-10 [105].

Other studies have shown that the protein formerly designated fibroblast-activating factor (FAF), previously characterized by Mihara and Holt [106] in *P. gingivalis* W50, W83, and ATCC 33277 strains, enhanced the proliferation of normal human gingival and skin fibroblasts, as well as umbilical vein endothelial cells. FAF induced higher levels of IL-6 production in human gingival fibroblasts compared to cells stimulated with *P. gingivalis* LPS and did not show action against human periodontal ligament cells [107, 108]. FAF has also been suggested to exert low phosphatase activity and participate in bone resorption. Interestingly, some amino acid sequences in peptides identified in FAF are identical to amino acid sequences in regions of the *P. gingivalis* HmuY protein [89].

The HmuY protein also seems to act in programmed cell death. PBMC stimulated with this protein appear to be unable to complete the process of apoptosis, resulting in death characterized by release of inflammatory cell content into the microenvironment, such as late apoptosis and necrosis, which can prolong the tissue destruction process [109]. Furthermore, the protein induces high levels of Bcl-2 in PBMC derived from individuals with chronic periodontitis, resulting in inhibition of apoptosis by enabling increased survival of T $CD3^+$ cells. All these findings strongly suggest that HmuY is one of the important virulence factors that allow effective *P. gingivalis* infection of host cells [93].

4. Conclusions

P. gingivalis, a "keystone pathogen" of chronic periodontitis, is an important component of the oral microbiome and a highly adapted colonizer. The bacterium has the ability to evade host defense systems and interfere in relationships between other oral species that make up the microflora located in supragingival and subgingival periodontal biofilm, leading to chronic inflammation, a cell prosurvival profile, and consequent tissue damage observed in individuals with chronic periodontitis. Molecules produced by *P. gingivalis* play an important role in the immunopathogenesis of chronic periodontitis, acting in both innate and adaptive immunity. One may conclude that the HmuY protein is important, at least in part, for efficient *P. gingivalis* growth in the heme-limited host environment, where the HmuY hemophore sequesters heme from host hemoproteins, thus allowing for efficient infection of host cells. In conclusion, *P. gingivalis* HmuY might play an important role in the immunopathogenesis of chronic periodontitis to evoke inflammatory responses, inhibit apoptosis, and interact with other bacterial species in biofilm formation (Figure 1). Therefore, the HmuY protein could be a promising target for therapeutic strategies

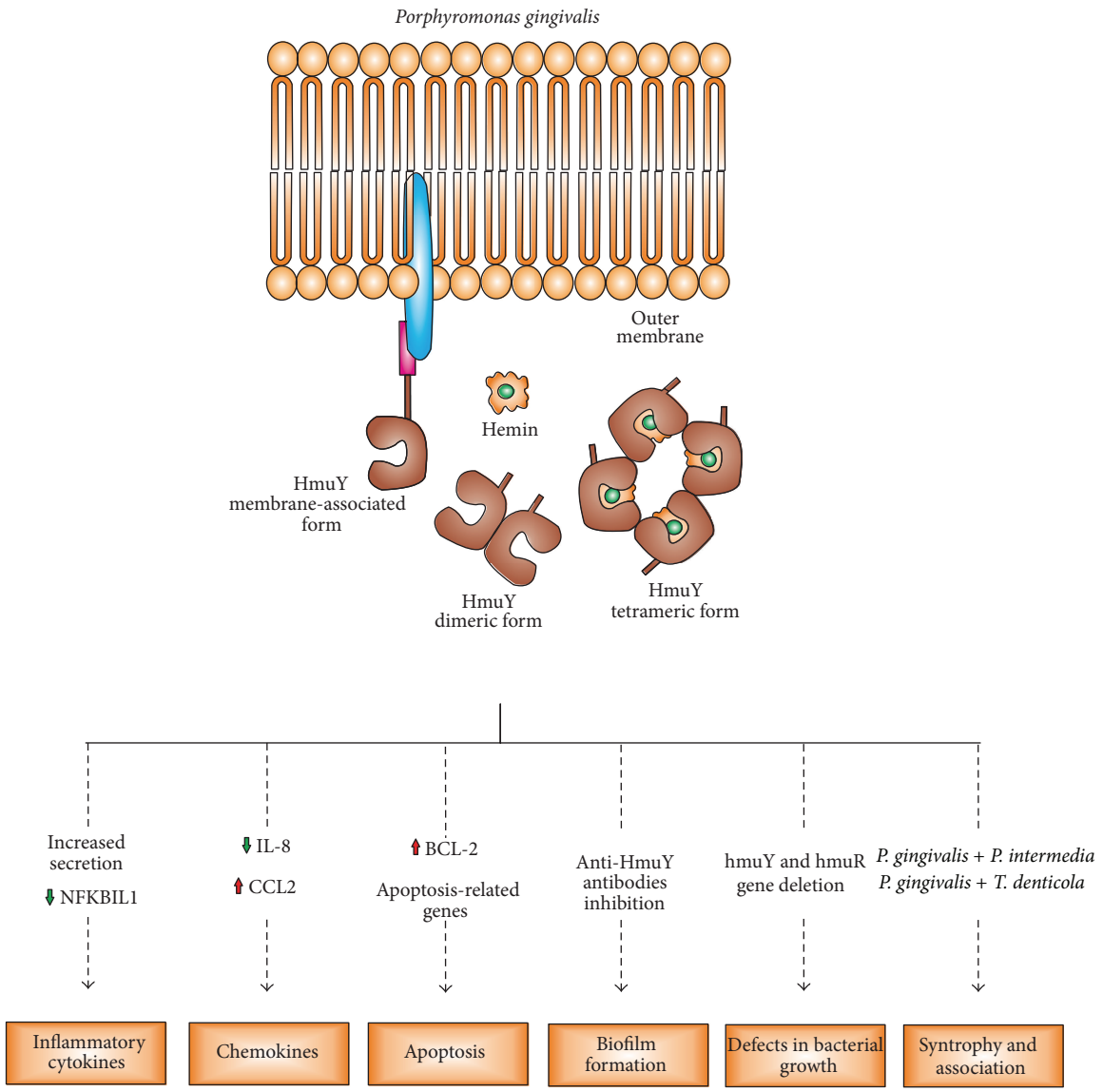

Figure 1: Schematic presentation of role of *P. gingivalis* HmuY in immunopathogenesis of chronic periodontitis.

and for diagnosis of chronic periodontitis which does not respond to periodontal treatment, as well as in monitoring of maintenance therapy in individuals with chronic periodontitis.

Competing Interests

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

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Research Article

Metallothioneins 1 and 2 Modulate Inflammation and Support Remodeling in Ischemic Cardiomyopathy in Mice

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Aims. Repetitive brief ischemia and reperfusion (I/R) is associated with left ventricular dysfunction during development of ischemic cardiomyopathy. We investigated the role of zinc-donor proteins metallothionein MT1 and MT2 in a closed-chest murine model of I/R. *Methods*. Daily 15-minute LAD-occlusion was performed for 1, 3, and 7 days in SV129 (WT)- and MT1/2 knockout ($MT^{-/-}$)-mice (n = 8–10/group). Hearts were examined with M-mode echocardiography and processed for histological and mRNA studies. *Results*. Expression of MT1/2 mRNA was transiently induced during repetitive I/R in WT-mice, accompanied by a transient inflammation, leading to interstitial fibrosis with left ventricular dysfunction without infarction. In contrast, $MT^{-/-}$-hearts presented with enhanced apoptosis and small infarctions leading to impaired global and regional pump function. Molecular analysis revealed maladaptation of myosin heavy chain isoforms and antioxidative enzymes in $MT1/2^{-/-}$-hearts. Despite their postponed chemokine induction we found a higher total neutrophil density and macrophage infiltration in small infarctions in $MT^{-/-}$-hearts. Subsequently, higher expression of osteopontin 1 and tenascin C was associated with increased myofibroblast density resulting in predominately nonreversible fibrosis and adverse remodeling in $MT1/2^{-/-}$-hearts. *Conclusion*. Cardioprotective effects of MT1/2 seem to be exerted via modulation of contractile elements, antioxidative enzymes, inflammatory response, and myocardial remodeling.

1. Introduction

The concept of brief repetitive episodes of myocardial ischemia and reperfusion (I/R) has been introduced as an important mechanism during development of ischemic heart disease [1]. Brief repetitive I/R episodes are not sufficient to induce myocardial infarction but are potent enough to trigger functional and morphological changes in the human heart resulting in a state of myocardial hibernation. This condition aims to preserve the myocardial integrity under conditions of impaired blood supply, that is, stenosis in coronary artery disease, at the cost of temporarily impaired myocardial function [2]. Upon restoration of a normal blood flow after revascularization therapy, the myocardial function recovers

within a six-month period [1]. This restoration of function has been associated with the presence of newly recruited leukocytes and low level of inflammation in myocardial segments with restoration of function. Based on these clinical findings we developed a murine model of brief repetitive I/R [3]. This model has a few characteristics comparable to the human hibernating myocardium: transient inflammatory reaction, reversible interstitial fibrosis, and reversible left ventricular dysfunction. We identified the presence of reactive oxygen species [3] and of the chemokine CCL2 [4] to be important for development of interstitial fibrosis without a loss of cardiomyocytes. In following studies we were able to depict mechanisms of cardioprotection and found an irreversible loss of cardiomyocytes and deteriorated cardiac function in mice deficient in endocannabinoid receptor CB2 [5] and in matricellular glycoprotein osteopontin 1 [6]. In both studies, the cardiomyocyte loss was associated with a significantly lower expression of metalloprotein MT1 and MT2, in contrast to their persistent induction in the WT-mice.

Induction of MT, a highly conserved zinc-storage protein, has been reported under several stress conditions involving the mechanisms of NO-mediated release of zinc [7] or inflammatory diseases [8]. Several studies utilized mice with cardiac-specific overexpression of metallothionein. One of them described attenuation of I/R injury in these mice [9], while another reports antiapoptotic effects after I/R [10]. A recent study provided evidence for attenuated myocardial remodeling in MT-overexpressing mice [11]. Few studies investigated the underlying mechanisms and reported association of MT with STAT3-mediated cardioprotection [12] and induction of Akt-pathway in prevention of cell death [13]. Primary culture of cardiomyocytes from mice deficient in MT1 and MT2 experienced increased toxicity and ROS generation after doxorubicin treatment [14]. Based on these results and our previous work, we postulated an important role for MT1/2 in cardioprotection and investigated the underlying mechanisms in MT1-/MT2-deficient mice during repetitive I/R.

2. Methods

2.1. Study Animals. We used transgenic mice with homozygous 129S7/SvEvBrd-*Mt1*^{tm1Bri} *Mt2*^{tm1Bri}/J mutation of the metallothionein 1 and 2 genes ($MT^{-/-}$) leading to a complete loss of their function [15]. These mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and compared to their background strain SV129 wildtype (WT)-mice, obtained from Charles River (Sulzfeld, Germany). All experiments were performed in mice of 20–25 g and 10–12 weeks of age. The study was in accordance with an animal protocol approved by the local governmental authorities and according to the EU Directive 2010/63/EU for animal experiments.

2.2. Brief, Repetitive I/R Procedure. For occlusion of the left descending coronary artery (LAD), an 8-0 Prolene suture (Ethicon, Norderstedt, Germany) was placed around it in an initial surgery and then stored subcutaneously as previously described [16]. Mice with initial surgery and 7-day recovery period without I/R were used as sham animals. After a 7-day recovery period the skin was reopened and occlusion of the LAD for 15 min with subsequent reperfusion were monitored in simultaneous ECG recording. This procedure was repeated for 1, 3, or 7 days as previously described and the hearts were excised five hours after the last ischemia episode [3]. Echocardiographic evaluation was performed in the 7-day group before heart retrieval. For histological experiments whole hearts were excised, rinsed with ice-cold cardioplegic solution, and fixated in zinc-paraformaldehyde (Z-fix, 4%; Anatech, Battle Creek, MI, USA). For mRNA studies hearts were further dissected free from great vessels and atria and immediately stored in RNA-later (Qiagen, Hilden, Germany) at 4°C [17].

2.3. Echocardiography. M-mode echocardiography in the parasternal short axis view at the level of the papillary muscle was performed using HDI-5000 system and a linear 15 MHz transducer (CL15-7; both ATL–Phillips, Oceanside, CA, USA) on anesthetized animals (2% isoflurane for induction and 0.8–1.2% in 100% O_2 for maintenance of anesthesia) [18]. Global left ventricular function was estimated by calculation of the fractional shortening (FS) and regional function of the left ventricular anterior wall via calculation of the anterior wall thickening (AWT) [19]. In order to estimate anesthesia-related cardiodepressive effects, the heart rate was monitored continuously and compared between both groups.

2.4. Basic Histology. Hearts were paraffinized and cut from basis to apex. Sections of 5 μm thickness from the papillary muscles insertion level were stained for basic histopathology (hematoxylin and eosin, HE) as already described [20]. Planimetric quantification of total collagen stained with picrosirius red (SR, Sigma-Aldrich, Steinheim, Germany) was presented as a percentage of the total left ventricular wall area, as already published [20]. Microinfarcted areas were related to the total collagen stained area and presented as a percentage.

2.5. Immunohistology. The following primary antibodies were used: alpha smooth muscle actin (α-SMAC) mouse monoclonal antibody (clone 1A4; Sigma, St. Louis, MO, USA), macrophages MAC-2 clone 3/38 rat antibody (AXXORA, Lörrach, Germany), neutrophils MCA771G (Ly-6B.2) clone 7/4 rat anti-mouse monoclonal antibody (MorphoSys, Oxford, UK), and tenascin C rabbit anti-chicken polyclonal antibody (Chemicon, Temecula, CA, USA). The following kits were used: M.O.M immunodetection kit for mouse-derived antibodies (AXXORA), Vectastain Elite ABC kits (Vector, Burlingame, CA, USA), and diaminobenzidine (AXXORA). Planimetric analysis of α-SMAC$^+$ area as percentage to anterior left ventricular wall was performed on two pictures with a magnification of 200x as published [20]. Cell density of macrophages and neutrophils was evaluated by manual cell count on four pictures (magnification 400x) taken from the anterior left ventricular wall [19].

2.6. Apoptosis Staining. Nuclei with DNA-fragmentation were stained with TUNEL In Situ Cell Death Detection Kit (POD, Roche, Mannheim, Germany) according to the manufacturer's protocol. The fluorescence signal was converted and then stained with diaminobenzidine (AXXORA) and slides were counterstained with Quick hemalaun kit (Vector) [19].

2.7. Gene Expression. The mRNA expression was measured with Taqman real time quantitative PCR system (RT-qPCR, Applied Biosystems, Foster City, CA, USA). Isolation of total RNA was performed with phenol/chloroform extraction (Trizol, Invitrogen). High capacity cDNA transcription kit with random hexameric primers (Applied Biosystems) was used for the synthesis of first-strand cDNA as described in the manufacturer's protocol. Taqman RT-qPCR was performed with 1/10 diluted cDNA on an ABI Prism 7900HT Sequence Detection System and SDS2.4 Software (Applied Biosystems). FAM-TAMRA® and the relative standard curve method were employed for the measurement of all primers. In order to ascertain the amplification of a single PCR product, dissociation curve analysis was performed. The mRNA expression was related to shams and GAPDH using comparative ΔΔCt-method [6, 19].

2.8. Statistical Analysis. Normal distribution of the data was tested and data presented as mean ± SEM. One-way ANOVA with Newman-Keuls *post hoc* testing using GraphPad PRISM (Version 5.0, Graphpad Inc., La Jolla, CA, USA) was performed. When comparing two groups, unpaired t-test was chosen. Differences with $P \leq 0.05$ were considered statistically significant.

3. Results

3.1. Deficiency in MT1 and MT2 Is Associated with Profound Left Ventricular Dysfunction and Cardiomyocyte Loss. Initially, we measured expression of MT1 and MT2 mRNA in WT-mice and found a transient upregulation of both during repetitive I/R (Figures 1(a) and 1(b)). The M-mode echocardiography measurements revealed a significantly lower fractional shortening and anterior wall thickening in both strains when compared to their respective shams (Figures 1(c)–1(e)). At the same time the $MT^{-/-}$-mice had a significantly worse fractional shortening than the WT-mice. HE staining showed no cellular infiltrations and intact myocardial structure in sham operated mice of both genotypes (Figures 1(f) and 1(h)). After 7 days of repetitive I/R an interstitial cellular infiltration in absence of cardiomyocyte loss was found in WT-hearts (Figure 1(g)) in contrast to $MT^{-/-}$-mice, which presented with cardiomyocyte loss in small, clearly delineated areas of infarction—microinfarctions—after 3 and 7 days of I/R (Figure 1(i)).

3.2. Apoptosis and Impaired Cellular Adaptation in $MT^{-/-}$-Mice. In the next step we further investigated the loss of cardiomyocytes using TUNEL staining for apoptosis. WT-mice showed only a few scattered TUNEL-positive nuclei in the ischemic myocardium after 3 days of I/R (Figure 2(a)). In contrast, $MT^{-/-}$-mice presented with a significantly increased number of apoptotic cells predominantly being found in microinfarctions and mostly having cardiomyocytes morphology (Figure 2(b)). Manual count of TUNEL-positive nuclei showed significantly higher apoptosis in $MT^{-/-}$-mice compared to WT after 3 days of I/R. (Figure 2(c)). The mRNA expression of related mediators revealed a comparable, nonsignificantly higher expression of caspase-8 in both genotypes after I/R (Figure 2(d)), while the caspase-3 was significantly downregulated in $MT^{-/-}$-hearts after 1 day of I/R (Figure 2(e)). Interestingly, $MT^{-/-}$-hearts presented also with a significant downregulation of antiapoptotic mediator B-cell lymphoma (Bcl)-2 at the same time (Figure 2(f)). These findings indicate a MT-related regulation of apoptosis mediators in the murine heart.

Since I/R leads to an excessive reactive oxygen species production, which may cause apoptosis of cardiomyocytes, we investigated expression of antioxidative mediators. Heme oxygenase (HMOX)1 mRNA expression was significantly higher in $MT^{-/-}$-mice when compared to WT after 3 days of I/R (Figure 3(a)). At the same time expression of glutathione peroxidase (GPX)1 was comparable between the genotypes (Figure 3(b)). The expression of superoxide dismutase (SOD) isoforms showed a significantly higher induction of SOD1 in WT-mice after 3 days of I/R (Figure 3(c)), no induction of SOD2 in both genotypes (Figure 3(d)), and significantly higher expression of SOD3 in WT-mice after 1 and 3 days of I/R (Figure 3(e)). In contrast, the expression of Ras-related C3 botulinum toxin substrate (Rac)1, the main regulator of NADPH oxidase, was significantly higher in $MT^{-/-}$-mice after 3 days of I/R (Figure 3(f)), indicating an at least partially preserved potential to quench the reactive oxygen species. Additionally, $MT^{-/-}$-mice presented with a significant downregulation of peroxisome proliferator-activated receptor (PPAR)-α after 3 and 7 days of I/R compared to sham (Figure 3(g)), indicating reduced utilization of fatty acids in order to further reduce oxidative stress burden [21]. Furthermore, the significant increase in uncoupling protein (UCP) 3 expression in $MT^{-/-}$-mice after 1 day of I/R versus respective WT-mice (Figure 3(h)) supports this assumption [22]. Another important mechanism triggering apoptosis in cardiomyocytes represents the maladaptation of contractile elements, that is, myosin heavy chain (MHC), during repetitive ischemic episodes [5]. We found a significant downregulation of the less energetically efficient α-MHC isoform accompanied by an upregulation of the β-MHC in WT-mice, as expected during adaptation to I/R (Figures 3(i) and 3(j)). In contrast, $MT^{-/-}$-mice presented with unchanged expression of the α-MHC accompanied by decreased expression of energetically more efficient β-MHC, a constellation indicative of a higher substrate consumption in their cardiomyocytes. Therefore, the increased loss of cardiomyocytes and apoptosis in $MT^{-/-}$-hearts seems to be associated with impaired regulation of antioxidative enzymes and contractile elements. Subsequently, these mice compensate the loss of cardiomyocytes by an increased cardiomyocyte size and hypertrophy (Figure 3(k)).

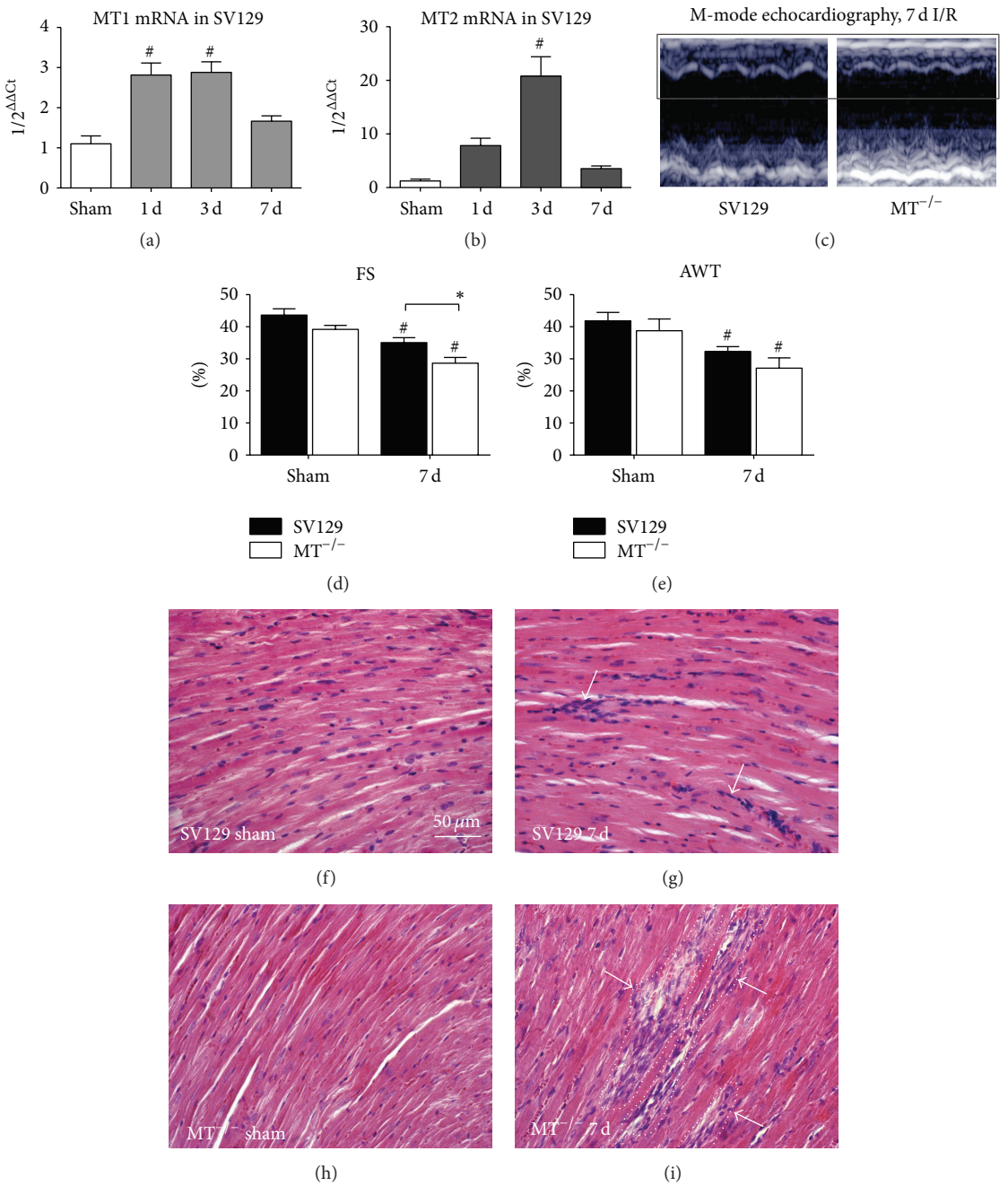

Figure 1: MT1-/2-deficiency leads to cardiomyocyte loss and deterioration of left ventricular function. The mRNA of (a) MT1 and (b) MT2 is upregulated after 1, 3, and 7 days (d) I/R in SV129 WT-mice. (c) Representative short axis M-mode echocardiographs from WT- and MT$^{-/-}$-mice after 7 days of (d) I/R. (d) and (e) reveal worse left ventricular function of MT$^{-/-}$-mice after 7 days of I/R compared to the WT. Representative HE-stainings reveal no cellular infiltration in both sham groups (f and h), but interstitial cellular infiltrates (arrows) into WT-hearts after 7 days of I/R (g), in contrast to microinfarcted areas with loss of cardiomyocytes (dotted line) and cellular infiltration (arrows) in MT1$^{-/-}$-mice at the same time (h and i). n = 8–10/group; scale bar in (f)–(i): 50 μm; RT-qPCR using Taqman®, mRNA expression is related to shams and GAPDH using comparative ΔΔCt-method; ∗ indicates $P \leq 0.05$ between the genotypes; # indicates $P \leq 0.05$ versus respective sham.

3.3. Inflammatory Response in Ischemic MT$^{-/-}$-Hearts. In order to investigate the potential of MT1 and MT2 to regulate inflammatory response we investigated migration of inflammatory cells into ischemic myocardium. Quantitative evaluation of MAC-2 positive macrophages showed a comparable cell density between the genotypes, which was significantly increased in both groups after 3 and 7 days when compared to their respective shams (Figures 4(a)–4(c)). Differential analysis of macrophage infiltration into ischemic area of WT-hearts revealed generally higher cell density in the interstitial

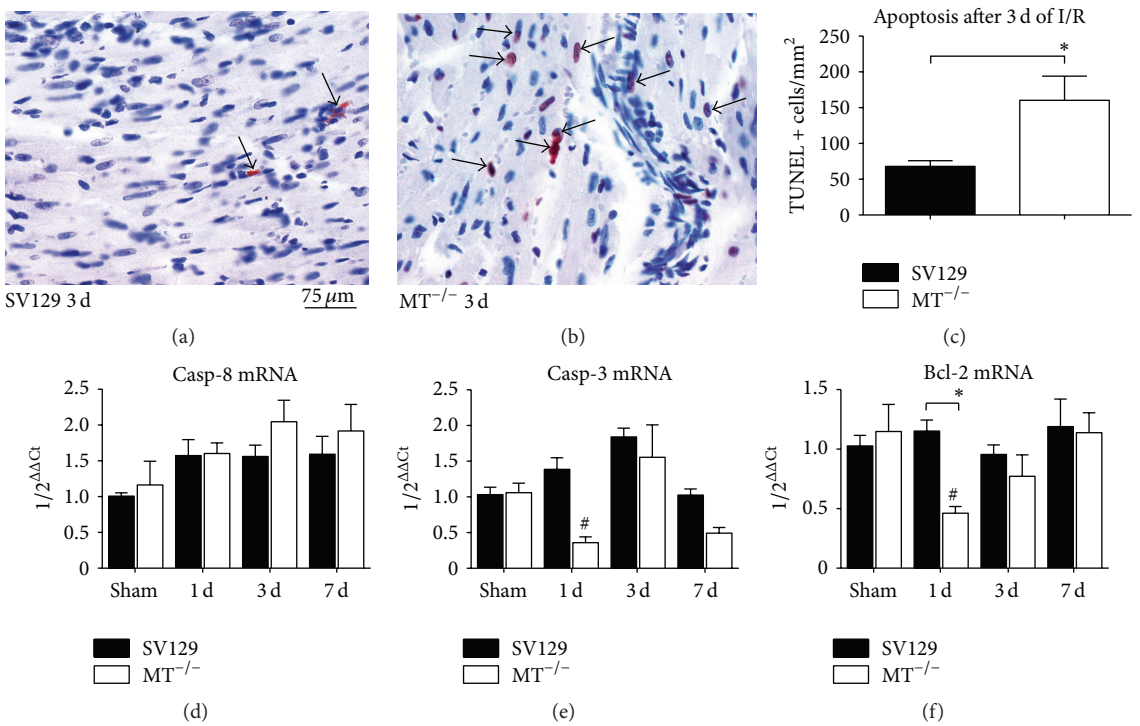

FIGURE 2: Increased cardiomyocyte apoptosis in MT$^{-/-}$-hearts. Representative slides of TUNEL stained sections present (a) only few apoptotic nuclei in cardiomyocytes of WT-hearts, when compared to (b) numerous nuclei with DNA-fragmentation in MT$^{-/-}$-hearts after 3 days of (d) I/R. (c) TUNEL-positive cells after 3 days of I/R. RT-qPCR showing mRNA expression of (d) caspase- (Casp-) 8, (e) caspase- (Casp-) 3, and (f) B-cell lymphoma (Bcl)-2 during repetitive I/R. n = 8–10/group; scale bar in (a) and (b): 75 μm; RT-qPCR using Taqman, mRNA expression is related to shams and GAPDH using comparative ΔΔCt-method; ∗ indicates $P \leq 0.05$ between the genotypes; # indicates $P \leq 0.05$ versus respective sham.

space than in the few small areas of cardiomyocyte loss and being significantly different after 3 days of I/R (Figure 4(d)). In contrast, a comparable macrophage density was found between interstitial space and microinfarctions in MT$^{-/-}$-mice during I/R (Figure 4(e)).

The analysis of MCA771G-positive neutrophils showed a significantly higher total cell density in MT$^{-/-}$-mice after 3 days of I/R (Figures 4(f)–4(h)). The differential analysis revealed only scattered neutrophils in WT-myocardium being located in the interstitial space (Figure 4(i)). At the same time, MT$^{-/-}$-mice presented with neutrophils located both in interstitial space and in microinfarctions (Figure 4(j)).

In the next step we measured the mRNA expression of chemokines and cytokines related to the migration of inflammatory cells into ischemic myocardium. The expression of the most potent macrophage chemoattractant CCL2 was significantly higher in WT- than in MT$^{-/-}$-hearts (Figure 5(a)). The chemokine CCL4 showed a similar expression pattern between the genotypes while having only one significant difference after 3 days of I/R (Figure 5(b)). Interestingly, at this time point MT$^{-/-}$-mice presented with a significant induction of CCL4 when compared to their shams. In addition, the expression of the neutrophil-related chemokine CCL3 followed the same pattern as the monocyte/macrophage-related CCL4 by having a significantly higher expression in WT-hearts compared to MT$^{-/-}$-hearts after 1 day of I/R, in contrast to a comparable induction after 3 days of I/R (Figure 5(c)). In contrast, the expression of the cytokine macrophage colony-stimulating factor (M-CSF) was again significantly higher in WT-mice, whereas MT$^{-/-}$-mice presented even with a significant downregulation of it when compared to their shams (Figure 5(d)). The expression of the cytokine TNF-α was also significantly higher during I/R when compared to MT$^{-/-}$-mice (Figure 5(e)). Still, the upregulation of the anti-inflammatory cytokine IL-10 was comparable between the groups, with only significant difference between 3 days of I/R and respective shams in MT$^{-/-}$-mice (Figure 5(f)). The expression of macrophage maturation marker osteopontin- (OPN-) 1 was significantly increased after 3 days of I/R in MT$^{-/-}$-mice (Figure 5(g)). Taken together, the MT$^{-/-}$-mice had a generally lower expression of proinflammatory chemokines and cytokines during I/R but also a preserved capability for resolution of inflammatory response and timely onset of myocardial remodeling.

3.4. Repetitive I/R *Leads to Adverse Myocardial Remodeling in MT$^{-/-}$-Mice.* Timely onset and intensity of myocardial remodeling is important in cardioprotection in order to limit the expansion of myocardial injury. The mRNA expression of remodeling-related transforming growth factor- (TGF-)

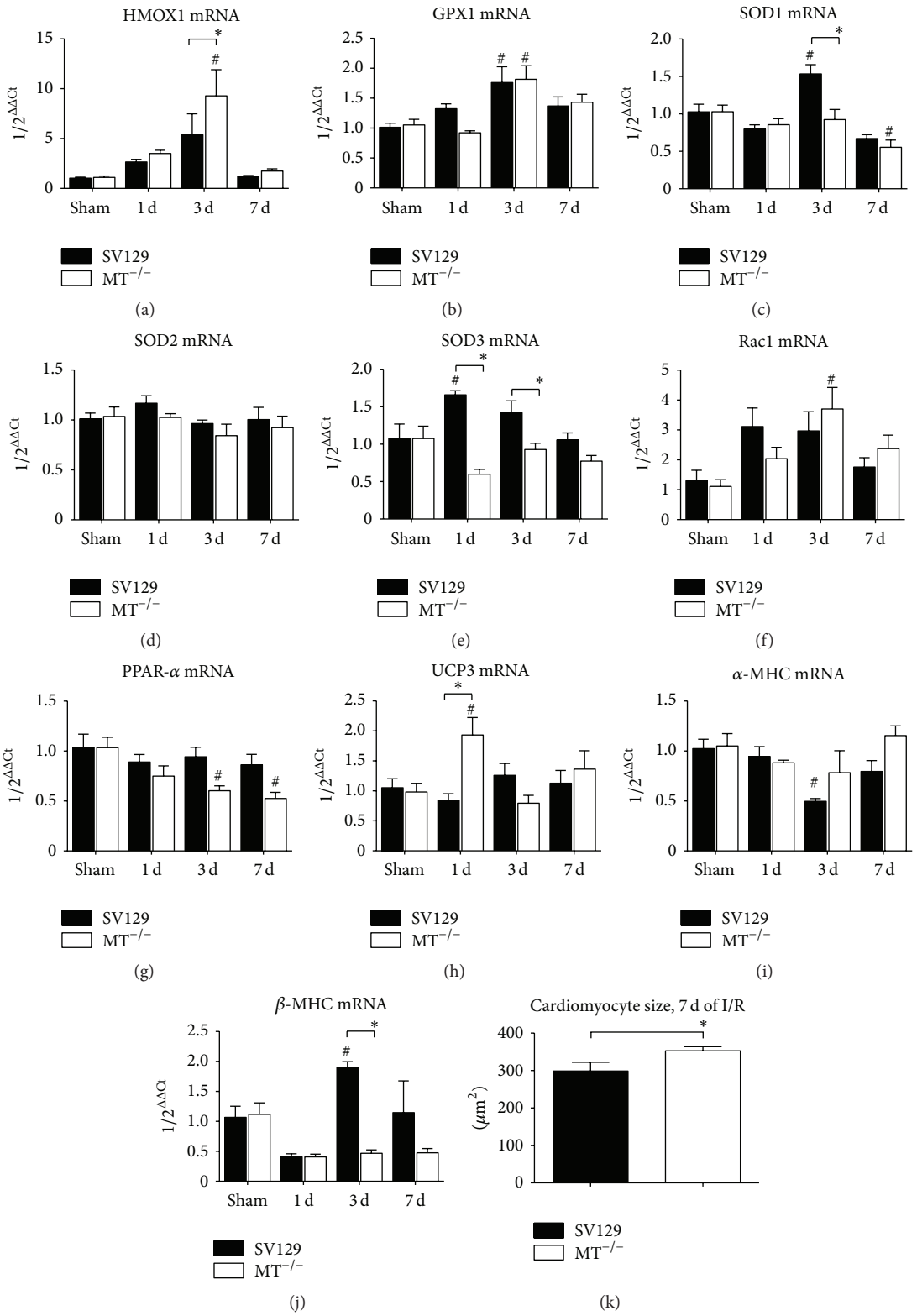

Figure 3: Impaired antioxidative control and maladaptation of contractile elements in $MT^{-/-}$-hearts. RT-qPCR showing mRNA expression of (a) heme oxygenase (HMOX)1, (b) glutathione peroxidase (GPX)1, (c) superoxide dismutase (SOD)1, (d) SOD2, (e) SOD3, (f) Ras-related C3 botulinum toxin substrate (Rac)1, (g) peroxisome proliferator-activated receptor (PPAR)-α, (h) uncoupling protein (UCP) 3, (i) myosin heavy chain (MHC) isoform α, and (j) β-MHC isoform. (k) Quantification of cardiomyocyte size by area planimetry on collagen stained slides after 7 days of I/R. n = 8–10/group; RT-qPCR using Taqman, mRNA expression is related to shams and GAPDH using comparative $\Delta\Delta$Ct-method; $*$ indicates $P \leq 0.05$ between the genotypes; # indicates $P \leq 0.05$ versus respective sham.

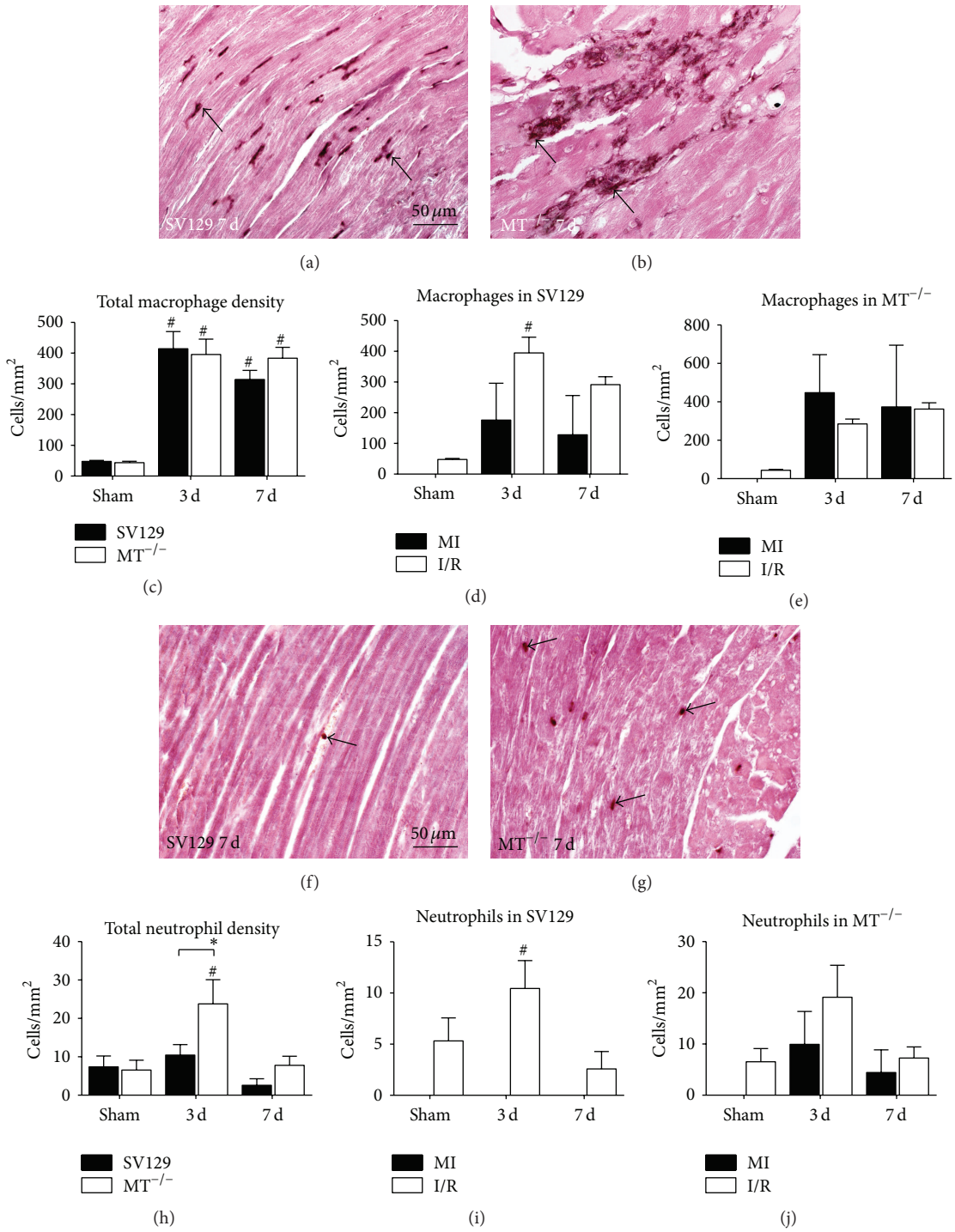

FIGURE 4: Macrophage and neutrophils infiltrate into areas of microinfarctions in $MT^{-/-}$-hearts. MAC-2 staining of representative left ventricular sections after 7 days of (d) I/R demonstrates predominant interstitial macrophage infiltration (arrow) in WT-hearts (a), in contrast to increased macrophage density in microinfarctions (arrows) in $MT^{-/-}$-hearts (b). (c) Quantification of MAC-2^+ cells shows increased macrophage infiltration after 3 and 7 days of (d) I/R in both genotypes. Differential analysis of macrophage accumulation revealed that macrophages were mainly located in the interstitial spaces in WT-hearts (d) but to an equal number in microinfarctions in $MT^{-/-}$-hearts (e). Representative MCA771G stained sections of WT- (f) and $MT^{-/-}$-hearts (g) after 7 days of I/R (arrows: neutrophils). (h) Quantification of MCA771G^+ cells showed higher neutrophil infiltration in $MT^{-/-}$-hearts after 3 days of I/R compared to WT. Neutrophil distribution was comparable to macrophages (i and j). n = 8–10/group; scale bars in (a), (b), (f), and (g): 50 μm; RT-qPCR using Taqman, mRNA expression is related to shams and GAPDH using comparative ΔΔCt-method; ∗ indicates $P \leq 0.05$ between the genotypes; # indicates $P \leq 0.05$ versus respective sham.

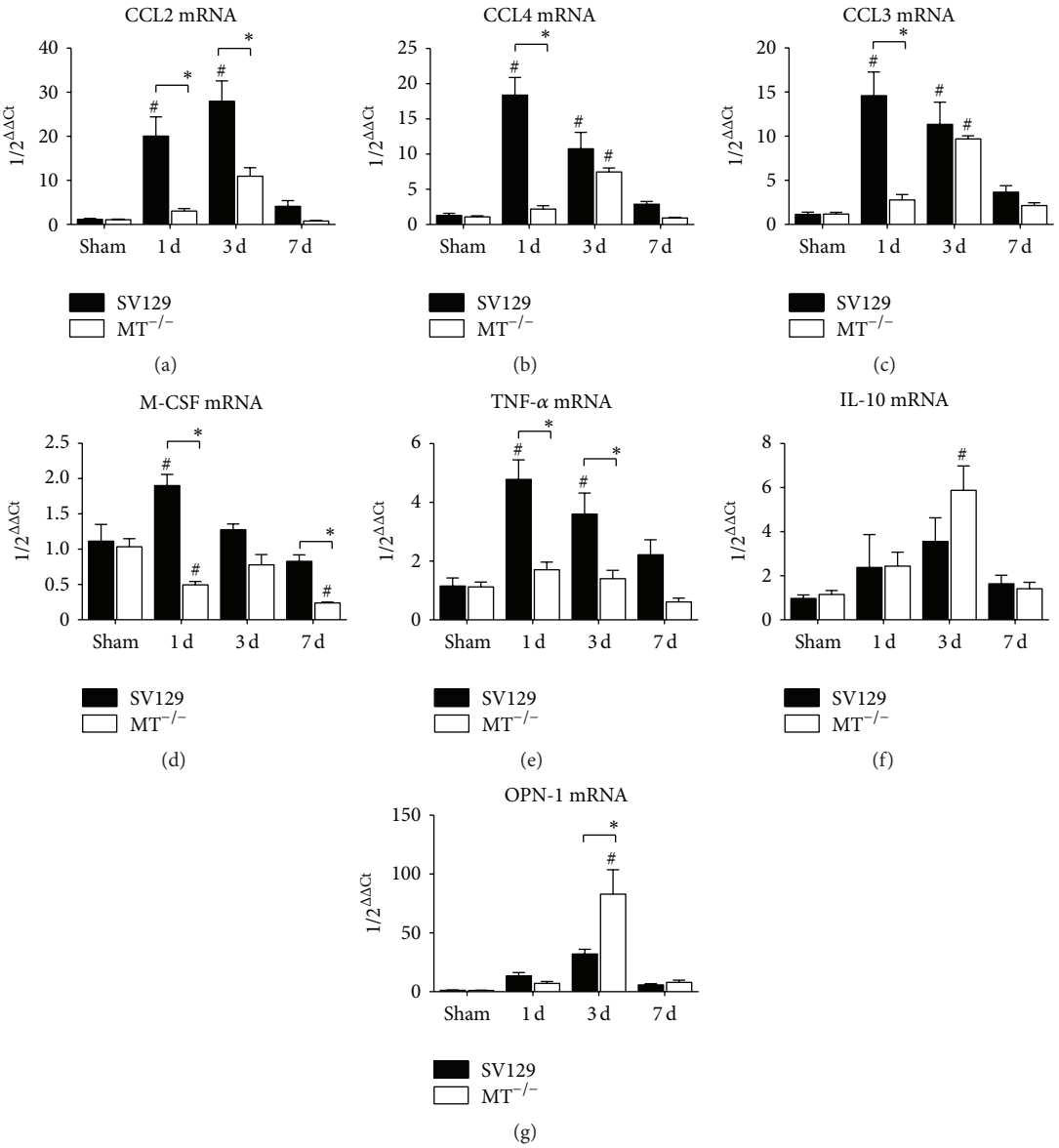

Figure 5: Gene expression of inflammatory mediators. Gene expression of macrophage-related chemokines (a) CCL2 and (b) CCL4 and (c) neutrophil-related CCL3 as well as cytokines (d) macrophage colony-stimulating factor (M-CSF), (e) TNF-α, (f) IL-10, and (g) osteopontin (OPN)-1. n = 8–10/group; RT-qPCR using Taqman, mRNA expression is related to shams and GAPDH using comparative $\Delta\Delta$Ct-method; $*$ indicates $P \leq 0.05$ between the genotypes; # indicates $P \leq 0.05$ versus respective sham.

β1 isoform showed a higher level in WT-mice being only significant after 1 day of I/R when compared to the MT$^{-/-}$-mice (Figure 6(a)). The β2 isoform of it showed no induction or difference between the genotypes (Figure 6(b)), while the β3 isoform presented with a significantly reduced expression after 1 day of I/R in MT$^{-/-}$-mice compared to WT (Figure 6(c)). WT-mice presented with scattered tenascin C (TNC) staining throughout the interstitial space of the ischemic area (Figure 6(d)). In contrast, the MT$^{-/-}$-mice revealed a significantly stronger staining signal (Figure 6(e)), as calculated upon its planimetric evaluation (Figure 6(f)). Similarly, WT-mice presented with a rather fine interstitial α-smooth muscle actin (α-SMAC) staining of myofibroblasts in contrast to a strong appearance of these cells in MT$^{-/-}$-mice (Figures 6(g) and 6(h)). Planimetric analysis of this staining revealed a significantly larger percentage of the total left ventricular area in MT$^{-/-}$-mice than in WT-mice (Figure 6(i)).

The higher intensity of myocardial remodeling seems to be attributable to the increased loss of cardiomyocytes and it resulted in significantly increased collagen area as a percentage of the total left ventricular area as well as microinfarctions in MT$^{-/-}$-mice (Figures 7(a)–7(c)). We also performed a differential analysis of collagen deposition and found significantly higher percentage of it in microinfarctions of MT$^{-/-}$-mice when compared to WT-mice (Figure 7(d)).

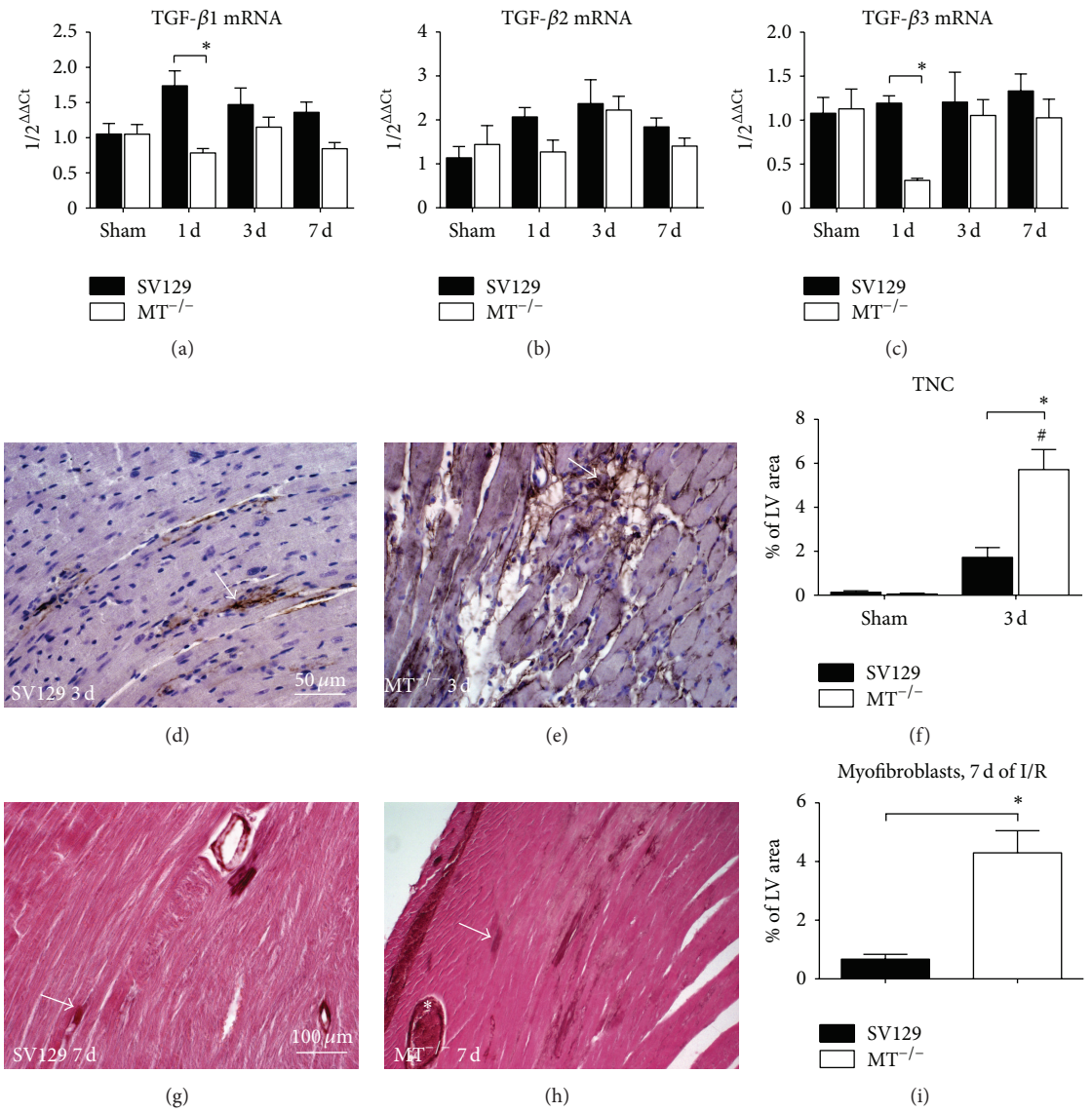

Figure 6: Adverse myocardial remodeling in $MT^{-/-}$-mice. Gene expression of remodeling-related transforming growth factor β isoforms (a) TGF-β1, (b) -β2, and (c) -β3. Representative tenascin C (TNC) staining of WT- ((d), arrow) and $MT^{-/-}$-hearts ((e), arrow) after 3 days of (d) I/R as well as the corresponding planimetric analysis (f). Representative left ventricular sections stained for myofibroblast marker α-smooth muscle actin (α-SMAC) after 7 days of I/R show (g) only few interstitial myofibroblasts in WT-hearts (arrow) but (h) strong myofibroblasts staining predominantly in microinfarctions (arrow) of $MT^{-/-}$-hearts after 7 days of I/R. (i) Planimetric analysis of α-$SMAC^{+}$ in both genotypes. n = 8–10/group; scale bars in (d) and (e): 50 μm, in (g) and (h): 100 μm; RT-qPCR using Taqman; mRNA expression is related to shams and GAPDH using comparative $\Delta\Delta$Ct-method; $*$ indicates $P \leq 0.05$ between the genotypes; # indicates $P \leq 0.05$ versus respective sham.

The mRNA expression of the reversible deposited collagen (Col)-III revealed a significantly higher level in WT- than in $MT^{-/-}$-mice, which practically showed downregulation of it (Figure 7(e)). As expected from histology we found significantly higher expression of the irreversibly deposited collagen (Col)-Iα form in $MT^{-/-}$-mice compared to WT (Figure 7(f)). Further analysis of matrix metalloproteinases (MMP) and their tissue inhibitors (TIMP) revealed a significantly higher expression of MMP-2 in WT-mice during I/R (Figure 7(g)). In contrast, the $MT^{-/-}$-mice presented with a significantly higher induction of MMP-9 after 3 days of I/R (Figure 7(h)). The expression of MMP-12 was comparable between the genotypes and here WT-mice showed a significant induction after 1 day of I/R when compared to their shams (Figure 7(i)). The expression of TIMP-1 showed a significantly higher induction after 3 days I/R in WT- compared to $MT^{-/-}$-mice, while both genotypes showed significant upregulation when compared to their respective shams at this time (Figure 7(j)). Interestingly, the $MT^{-/-}$-mice presented with a significantly higher induction of TIMP-4

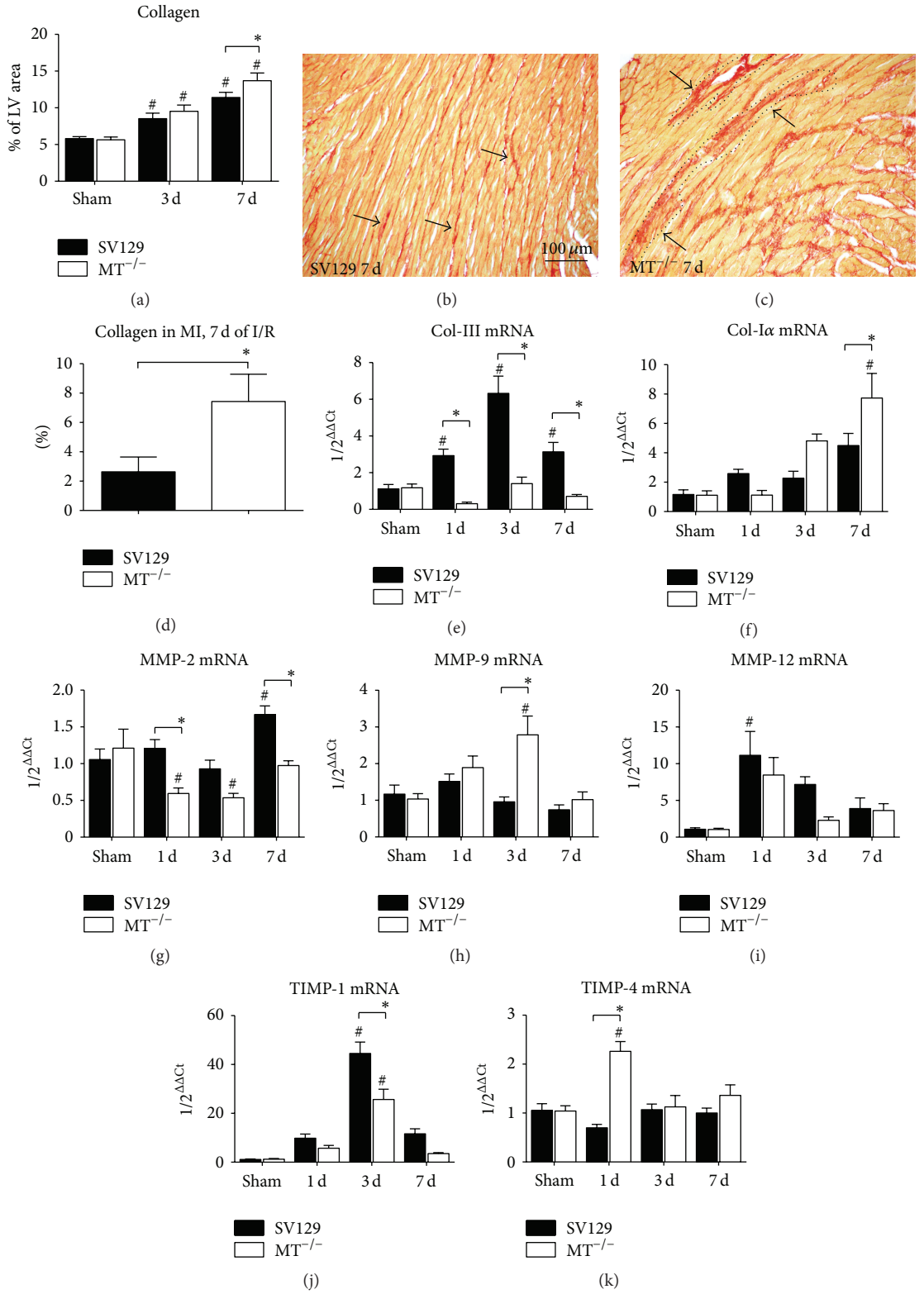

FIGURE 7: Scar formation and inadequate extracellular matrix turnover in MT$^{-/-}$-mice. (a) Planimetric analysis of picrosirius red stainings shows increasing total collagen area after 3 and 7 days (d) in both genotypes. Representative picrosirius red stainings after 7 days of I/R shows (b) interstitial fibrosis (arrows) in WT-heart, in contrast to (c) dense collagen deposition in microinfarctions (arrows, dotted line: microinfarcted area) in MT$^{-/-}$-hearts. (d) Significantly higher collagen deposition in microinfarcted areas in MT$^{-/-}$-hearts after 7 days of I/R compared to WT. Evaluation of mRNA expression of (e) collagen (Col)-III, (f) Col-Iα, (g) metalloproteinase (MMP)-2, (h) MMP-9, and (i) MMP-12 as well as (j) tissue inhibitor of MMP (TIMP)-1 and (k) TIMP-4. n = 8–10/group; scale bars in (b) and (c): 100 μm; RT-qPCR using Taqman, mRNA expression is related to shams and GAPDH using comparative $\Delta\Delta$Ct-method; $*$ indicates $P \leq 0.05$ between the genotypes; # indicates $P \leq 0.05$ versus respective sham.

after 1 day I/R compared to WT and shams (Figure 7(k)). We also calculated the ratio of MMP-9 to TIMP-1 mRNA expression and found a significantly lower ratio of 0.02 in WT-hearts when compared to 0.11 in $MT^{-/-}$-hearts after 3 days of I/R. The ratio of MMP-2 to TIMP-4 presented with a significantly higher ratio of 1.73 in WT-hearts when compared to 0.26 in $MT^{-/-}$-hearts after 1 day of I/R. Taken together, $MT^{-/-}$-mice experienced an intense myocardial remodeling with more collagen deposition and imbalanced expression of MMP and TIMP in the ischemic area when compared to WT-mice.

4. Discussion

Previous studies utilized cardiac-specific overexpression of metallothionein to introduce its involvement in cardioprotective mechanisms [23]. One of them utilized an *ex vivo* model of reperfused infarction to show a reduced infarction size [9]. Another study used an *in vivo* model of coronary occlusion followed by four hours reperfusion and suggested that the protection of metallothionein excess against cardiomyocyte apoptosis is mediated via reduction of reactive oxygen species [10]. We have chosen a different approach to characterize the role of metallothionein in myocardial ischemia by using $MT^{-/-}$-mice in a model of brief repetitive I/R without myocardial infarction. This nonlethal ischemia model is of clinical relevance in regard to prevention and early therapy before myocardial injury occurs [2]. Our data provide not only further evidence for cardioprotective effects of metallothionein but also describe downstream effects on mediators during adaptation, inflammatory response, and myocardial remodeling in ischemic, noninfarcted cardiomyopathy. Furthermore, we provide evidence that the wild type SV129 mouse strain shares the same pattern of functional, morphological, and molecular events after repetitive I/R as the previously published C57/Bl6 strain [3, 5].

The murine model of brief repetitive I/R leads to a transient inflammatory reaction involving predominately chemokine induction and macrophage migration into ischemic myocardium [3]. This results in an interstitial fibrosis and left ventricular dysfunction, which are both reversible after discontinuation of the I/R protocol. Our current study provides detailed characterization of these events in SV129 mouse strain for the first time. We could show a very much comparable course of chemokine and cytokine induction, macrophage migration, and remodeling-related factors in comparison to the C57/Bl6 mouse strain. Also, the extent of interstitial fibrosis and left ventricular dysfunction are at the same level in both wild type mouse strains. This is important not only for interpretation of the $MT^{-/-}$-mice data but also for future studies using this model in other genetically manipulated mice.

The $MT^{-/-}$-mice experienced an irreversible loss of cardiomyocytes already after 3 days of I/R and this pathology became clearly delineated after 7 days of I/R. Even though we found no significant difference in regional pump function of the anterior left ventricular wall between the genotypes after 7 days of I/R, there was a significantly worse global left ventricular function in $MT^{-/-}$-mice at the same time. Our data provide evidence for cardiomyocyte loss—at least in part by apoptosis—in the lack of MT1 and MT2 and thereby confirm the previously published association with apoptosis [10, 24]. In addition, our data on apoptosis related mediators suggest a link between expression of metallothionein and the antiapoptotic Bcl2 in prevention of cardiomyocyte apoptosis. This is in concordance with the reported role of metallothionein in Akt-pathway as cardioprotective mechanism [13]. Also, our data support the previously published association of metallothionein with antioxidative enzymes in myocardial injury [10]. Our findings indicate a decreased induction of antioxidative enzymes in $MT^{-/-}$-mice but a preserved activation of Rac1 and thereby induction of NADPH oxidase. Since activation of Rac1 has been associated with Notch2 regulation for neuroprotection after I/R injury [25], we assume the contribution of this effect to prevent even a higher loss of cardiomyocytes in $MT^{-/-}$-mice. Another effect aiming at further reduction of reactive oxygen species production is indicated by downregulation of PPAR-α in $MT^{-/-}$-mice, resulting in decreased utilization of fatty acids [19, 21]. This is further supported by upregulation of UCP3 in $MT^{-/-}$-mice, which represents an additional mechanism to counteract the high burden of free radicals [22]. Therefore, the published data and our findings strongly indicate a redundancy in these mechanisms in order to prevent detrimental loss of cardiac function. While other studies provided evidence for the association of metallothionein with STAT-3 [12] and Akt-pathway [13] during cardioprotection, we investigated the contractile elements of cardiomyocytes. $MT^{-/-}$-mice presented with malfunction in adaptation of α- and β-MHC resulting in high substrate consumption, which is also contributing to increased cardiomyocyte apoptosis [5, 21]. In consequence, the remaining viable cardiomyocytes presented with hypertrophic response in $MT^{-/-}$-mice and one can therefore speculate that the continuation of repetitive I/R beyond 7 days would cause further loss of cardiomyocytes.

The production of reactive oxygen species leads to inflammatory reaction during repetitive I/R. Our data show a clear dependence of initiation of chemokine induction on the presence of metallothionein in this model. This may be specific to the low level, nonbacterial inflammation in noninfarcted heart, since a study reported that *Helicobacter pylori* infected MT-null mice (C57/Bl6 background) have a significantly higher induction of CCL2 [26]. The chemokines took the lead in $MT^{-/-}$-hearts to initiate an inflammatory reaction after 3 days of I/R and attract macrophages and neutrophils, whereas the cytokine expression was even downregulated. The higher neutrophils density in $MT^{-/-}$-hearts is clearly associated with the cardiomyocyte apoptosis and the need for debris removal in these hearts when compared to the very faint loss of cardiomyocytes in WT-mice without microinfarctions. The higher migration of inflammatory cells into ischemic hearts of $MT^{-/-}$-mice is still associated with a timely resolution of inflammatory response, as shown by the induction of anti-inflammatory cytokine IL-10 and a macrophage maturation marker OPN-1 [27]. This suggests

a rapid transition to myocardial remodeling in order to preserve myocardial function [1].

Interstitial fibrosis represents the hallmark of myocardial remodeling during repetitive I/R. Our findings showed the lack of induction or even downregulation of the expression of two transforming growth factor β isoforms TGF-β1 and -β3 after 1 day of I/R, which may be associated with the initiation of a strong remodeling response. Our data revealed first evidence for metallothionein-related deposition of TNC in ischemic murine hearts. Since TNC is related to early stages of tissue remodeling and embryonic development [28], its stronger production in $MT^{-/-}$-mice can be associated with significantly increased differentiation of myofibroblasts in their hearts. This results in a higher intensity of myocardial remodeling due to the lack of metallothionein, which is in accordance with the previously published data on attenuated myocardial remodeling in MT-overexpressing mice [11]. Therefore, metallothionein seems also to be directly involved in modulation of myocardial remodeling, but we can only speculate on related mechanisms. The analysis of collagen deposition and production revealed practically a lack of the reversible collagen III induction in $MT^{-/-}$-mice. This seems be associated with the increased need for deposition of stable collagen Iα in the scars of microinfarctions in these mice, as further indicated by its strong mRNA induction. The data on MMP- and TIMP-expression also support our interpretation of formation of nonreversible collagen deposition during scar formation in $MT^{-/-}$-mice. The MMP/TIMP ratio also indicates a shift in balance between production of extracellular matrix components and adverse remodeling in $MT^{-/-}$-hearts.

We did not investigate the reversibility of the phenotype in $MT^{-/-}$-mice since our previous studies showed permanently impaired function and scar persistence 60 days after discontinuation of the I/R in mice with microinfarctions [5]. Also, the interpretation of our findings may have a weakness which is that it is only based on mRNA expression without protein data. Still, the latter is relative since most of the mediators we investigated are transcriptionally well regulated.

5. Conclusions

In conclusion, metallothionein seems to provide cardioprotection via modulation of antioxidative enzymes and contractile elements, regulation of inflammatory response, and subsequent myocardial remodeling in murine model of ischemic cardiomyopathy without infarction. Our findings may open a therapeutic perspective for targeting metallothionein in prevention or treatment of ischemic heart disease.

Competing Interests

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

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Clinical Study

Effects of ACL Reconstructive Surgery on Temporal Variations of Cytokine Levels in Synovial Fluid

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Anterior cruciate ligament (ACL) reconstruction restores knee stability but does not reduce the incidence of posttraumatic osteoarthritis induced by inflammatory cytokines. The aim of this research was to longitudinally measure IL-1β, IL-6, IL-8, IL-10, and TNF-α levels in patients subjected to ACL reconstruction using bone-patellar tendon-bone graft. Synovial fluid was collected within 24–72 hours of ACL rupture (*acute*), 1 month after injury immediately prior to surgery (*presurgery*), and 1 month thereafter (*postsurgery*). For comparison, a "control" group consisted of individuals presenting chronic ACL tears. Our results indicate that levels of IL-6, IL-8, and IL-10 vary significantly over time in reconstruction patients. In the *acute phase,* the levels of these cytokines in reconstruction patients were significantly greater than those in controls. In the *presurgery* phase, cytokine levels in reconstruction patients were reduced and comparable with those in controls. Finally, cytokine levels increased again with respect to control group in the *postsurgery* phase. The levels of IL-1β and TNF-α showed no temporal variation. Our data show that the history of an ACL injury, including trauma and reconstruction, has a significant impact on levels of IL-6, IL-8, and IL-10 in synovial fluid but does not affect levels of TNF-α and IL-1β.

1. Introduction

Anterior cruciate ligament (ACL) injuries represent approximately 25% of all knee injuries with an annual incidence of at least 0.8 per 1000 persons aged between 10 and 64 years [1]. Nowadays more than 175,000 ACL reconstructions are performed in the US annually [1, 2]. ACL reconstruction can provide knee stability, permit continued participation in sports, and reduce the incidence of other intra-articular knee injuries, such as meniscal and chondral lesions [3–6]. An association between delaying ACL surgery and the development of subsequent meniscal tears and chondral lesions has been well documented in adults and skeletally immature patients [5, 6]. This consequence could depend on an excessive anterior tibial translation and a rotational instability in the injured knee. Presently, graft choices for primary ACL reconstruction include patellar tendon-bone (BPTB) and hamstring (HT) autografts [7]. Even though BPTB autografts may be better in restoring stability than HT autografts, knee-joint stability restored by ACL reconstruction does not decrease the incidence of posttraumatic osteoarthritis (OA) [4, 7, 8]. Fifty to 60% of patients with ACL-reconstructed knees have radiographic evidence of OA after five years [8, 9]. This observation suggests the importance of other factors in the pathophysiology of posttraumatic OA after ACL injuries, including intra-articular inflammatory reactions. High levels of inflammatory cytokines (interleukins, ILs) such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and

tumor necrosis factor-α (TNF-α) have been detected in acute and chronic anterior cruciate ligament injured knees, suggesting that cytokines can promote cartilage catabolism through the synthesis of free radicals and metalloproteases (MMPs) and consequently participate in OA development [9–11]. Biochemical changes in the synovial fluid of ACL-damaged knees have been extensively reported, but the evolving joint biochemical processes are not clearly documented [12, 13]. These studies examined cytokines or MMPs in the knee joint in a specific clinical situation such as acute or chronic injury but did not follow these levels longitudinally in individual patients undergoing ACL reconstruction. As there are large variations in absolute levels of synovial fluid cytokines among similarly compromised patients [9, 10], it is critical to track individual patients longitudinally to determine if there are different patient-specific cytokine responses.

In this study, we examined knee joint inflammatory patterns in each patient (i) within 24–72 hours of ACL rupture (*acute*), (ii) 1 month after injury immediately prior to arthroscopic ACL reconstruction surgery (*presurgery*), and (iii) 1 month thereafter (*postsurgery*). In particular, we measured the concentrations of selected pro- and anti-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, and TNF-α) in the synovial fluid. For comparison, a corresponding "control" group consisted of individuals ($n = 17$) presenting chronic ACL tears. The aim of the present study was to better understand how cytokines profiles evolve in the same patient after ACL rupture and after ACL reconstruction with BPTB.

2. Methods

2.1. Participants. Eight male participants aged between 18 and 40 years (mean: 24.5±7.5 years) presenting ACL injury within 72 hours were recruited by an orthopedic surgeon for the periacute ACL tear group. Inclusion criteria consisted of an isolated ACL tear, or an ACL tear associated with a meniscal tear (not itself requiring surgical treatment) confirmed by a senior orthopedic surgeon using present history, physical examination (including positive Lachman and Pivot Shift tests), magnetic resonance imaging, and a confirmation by arthroscopic examination. In comparison, 17 male patients (mean age: 32.6 ± 9.05 years; 5 patients with isolated ACL tear and 12 patients with associated meniscal tear) were recruited from the local community and selected for a chronic ACL tear (3 months or more from the trauma as previously defined) confirmed clinically, with a MRI and arthroscopic surgery [11]. These chronic ACL tear subjects were pooled as concentrations of IL-1β, IL-6, IL-8, IL-10, and TNF-α are independent of associated meniscal injury [11]. Individuals were excluded from either group if they had a previous history of knee injury, bone fractures simultaneously at investigated knee sprain, chondral lesion or chondropathy with a grade ≥ II according to Outerbridge classification, inflammatory arthritis, osteochondral lesion, and previous intra-articular injection of steroid or hyaluronic acid or NSAID treatment.

Synovial fluid was collected from participants of the periacute ACL tear group 3 times: (i) *acute*: 24–72 hours from the ACL rupture, (ii) *presurgery*: 1 month after the acute event (when an arthroscopic ACL reconstruction surgery was planned); and (iii) *postsurgery*: 1 month after the surgery. Arthroscopic ACL reconstruction was performed using bone-patellar tendon-bone (BTB) graft by the senior surgeon (Marco Bigoni). Femoral fixation was performed with two bioabsorbable cross-pins using RigidFix (DePuy Mitek, Raynham, MA, USA) and tibial fixation with a bioabsorbable interference screw (9 × 23 mm; DePuy Mitek, Raynham, MA, USA). After surgery, all patients followed the same accelerated rehabilitation protocol focusing on quadriceps recruitment and early gain of full range of motion. In the chronic ACL group, synovial fluid was drawn at the beginning of arthroscopic surgery.

Protocols were approved by the local Human Research Ethical Committee and conformed to the principles outlined in the WMA Declaration of Helsinki. All participants provided written informed consent.

2.2. Samples. Synovial fluid was aseptically collected without lavage at the beginning of arthroscopic surgery or during initial clinical reception in the emergency room. Synovial fluid samples, collected in tubes containing EDTA, were immediately centrifuged at room temperature (3,000 g × 10 min) in order to remove cellular debris and the supernatant was stored at −80°C until being assayed [10, 11]. The levels of interleukins (IL-1β, IL-6, IL-8, and IL-10) and tumor necrosis factor (TNF-α) were measured using specific sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (IL-1β, IL-10, and TNF-α, R&D Systems, Minneapolis, MN; IL-6 and IL-8, eBioscience, San Diego, CA). Detection limits were 2.2 pg/mL, 0.8 pg/mL, 0.5 pg/mL, 2 pg/mL, and 1 pg/mL, respectively, for IL-6, IL-8, TNF-α, IL-10, and IL-1β.

2.3. Statistical Analysis. Statistical analysis was performed using MATLAB (version R2010b; MathWorks). Normality of data distribution was assessed by the Jarque-Bera test. Since data were not normally distributed, nonparametric tests were performed.

When comparing temporal patterns, the Kruskal-Wallis test for statistical difference between 3 groups (i.e., *acute*, *presurgery*, and *postsurgery*) was performed, which is a nonparametric version of classical one-way ANOVA. In the case of significance, Mann-Whitney test Bonferroni corrected has been performed as *post hoc* analysis. When comparing the periacute ACL tear group of patients and the chronic ACL tear group in the different temporal sampling, the Mann-Whitney test was used, which is the nonparametric formulation of the t-test, Bonferroni corrected for multiple comparison. For all statistical tests, p values < 0.05 appropriately corrected for multiple comparison where needed were considered to be statistically significant.

For all statistical tests, since they were Bonferroni corrected for multiple comparisons, values of $p < 0.0167$ were considered to be statistically significant.

3. Results

3.1. Effects of Trauma on Cytokine Levels in Synovial Fluids. Measurement of cytokines levels in samples obtained shortly

Table 1: Cytokine levels in synovial fluids from longitudinal ACL group and from chronic ACL tear group.

	IL-6 (pg/mL)	IL-8 (pg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	IL-1β (pg/mL)
Longitudinal ACL group					
Acute samples	612.19 ± 124.98*	362.3 ± 164.60*	1.35 ± 0.52	29.09 ± 1.19*	9.07 ± 5.18
Presurgery samples	163.34 ± 106.35	38.22 ± 26.05	2.47 ± 0.56	8.82 ± 2.05	12.94 ± 6.35
Postsurgery samples	855.37 ± 183.12*	407.48 ± 109.71*	2.4 ± 0.48	11.21 ± 2.03*	9.42 ± 3.07
Chronic ACL tear group					
Samples	58.52 ± 5.86	23.66 ± 1.55	6.15 ± 0.45	4.03 ± 0.21	6.15 ± 0.81

Cytokine concentrations (pg/mL) in the synovial fluid of patients of the longitudinal group ($n = 8$) and chronic ACL tears ($n = 17$). Samples of longitudinal group were further assigned to groups according to the moment of the synovial fluid collection: *acute* samples (less than 72 hours after ACL injury); *presurgery* samples (1 month after the trauma, just before the arthroscopic ACL reconstruction surgery); *postsurgery* samples (1 month after the surgery). Values are expressed as mean ± standard error of the mean (SEM). *Statistically significant periacute samples in comparison with chronic ACL tear group.

after ACL tear confirms the picture of an inflammatory acute reaction. In fact, levels of IL-6 were significantly higher ($p < 0.001$) compared to the chronic ACL tear group (Table 1). Similarly, also IL-8 levels were significantly higher ($p < 0.001$) in the acute samples compared to the chronic ACL tear group (Table 1). Compared to chronic ACL group, IL-8 and IL-6 levels were elevated 15- and 11-fold, respectively (Table 1). IL-10 levels were also increased but to a lesser extent ($p < 0.001$), whereas IL-1β and TNF-α levels were comparable in acute samples and chronic ACL group (Table 1).

3.2. Temporal Patterns. Surgical trauma for ACL reconstruction clearly stimulated a recurrence of the inflammatory picture, causing IL-6, IL-8, and IL-10 concentrations to follow a "V-shaped" trend (i.e., high-low-high values) (Figure 2). Indeed, *acute* and *postsurgery* IL-6, IL-8, and IL-10 levels result in being significantly higher with respect to the chronic ACL tear group (Figure 2). In the presurgery phase, synovial fluid levels of IL-6, IL-8, and IL-10 displayed a rapid decrease (Figure 1), so that 30 days after injury they were in the range of those measured in chronic ACL group (Table 1). IL-1β and TNF-α levels in each sample of longitudinal group were similar with respect to chronic ACL tear group at any time (Figure 2) and did not follow a specific trend correlated with time after injury (Figure 1). In particular, *post hoc* analysis demonstrated that in the *presurgery* assessment IL-6 and IL-8 concentrations are significantly lower with respect to *acute* assessment ($p = 0.0111$), and in *postsurgery* assessment their levels significantly increased ($p = 0.0111$; $p = 0.0041$). Similarly, we measured a significant decrease of IL-10 levels from the *acute* to *presurgery* samples ($p < 0.001$).

4. Discussion

Acute ACL injuries establish an inflammatory reaction that persists chronically after resolution of the acute effusion [10, 14, 15]. Knee-joint inflammatory cytokines can promote cartilage catabolism through the synthesis of free radicals and metalloproteases and develop OA [16, 17]. Moreover, cytokine-mediated inflammatory responses could play an important role in bone tunnel enlargement following ACL reconstruction.

Cytokines as IL-6, IL-8, and IL-1β promote osteoclastic activity and can contribute to bone resorption [18]. In a rabbit model, it was described that interarticular bone tunnel healing was slower and less complete in the articular part of the tunnel, suggesting an important role of the synovial environment in the graft integration [19].

Different studies reported high pathological levels of cytokines in the synovial fluid after ACL injury, chronically, and after ACL reconstruction, but to our knowledge the biological natural history of an ACL tear with multiple synovial fluid collections remains uninvestigated [9, 10, 13].

IL-6 and IL-8 are proinflammatory cytokines with an important role in cartilage and bone damage. IL-6 in the joint environment reduces the production of type II collagen, increases the production of MMPS, and is considered to be the key cytokine in the subchondral bone degradation [17, 20]. Moreover, *in vitro* models showed that IL-6 role in the cartilage destruction is markedly potentiated by mechanical injury [21]. IL-6 has the capability to increase the production of inflammatory chemokines, such as IL-8 in synoviocytes and monocytes [22].

IL-8 is a potent chemokine with a key function in the promotion of neutrophil-mediated inflammation and cartilage destruction [23]. These two cytokines present a specific and similar trend in response to the articular events. Immediately after the ACL rupture, IL-6 and IL-8 levels largely increased compared to the chronic ACL tear group. Interestingly, 1 month after IL-6 and IL-8 levels decreased while remaining higher than in the chronic control group. The ACL reconstruction, the presence of foreign bodies and allograft, and the accelerated rehabilitation protocol caused postsurgery levels of IL-6 to increase more than fivefold compared to the presurgery levels. Similarly, also postsurgery levels of IL-8 increased more than tenfold compared to the presurgery levels. This could suggest a role for IL-8 and IL-6 not only in the very early phase of joint inflammation but in all the biological history of a knee with an ACL injury and reconstruction.

IL-10 is a modulator cytokine that contributes to the suppression of the inflammation of the synovial membrane and is endowed with chondroprotective properties. It stimulates the synthesis of type II collagen and aggrecan and antagonizes the release of MMP in chondrocyte [17]. We found high levels of IL-10 in *acute* samples compared to chronic ACL tear group as previously reported [11]. One month later, IL-10 levels decreased more than threefold

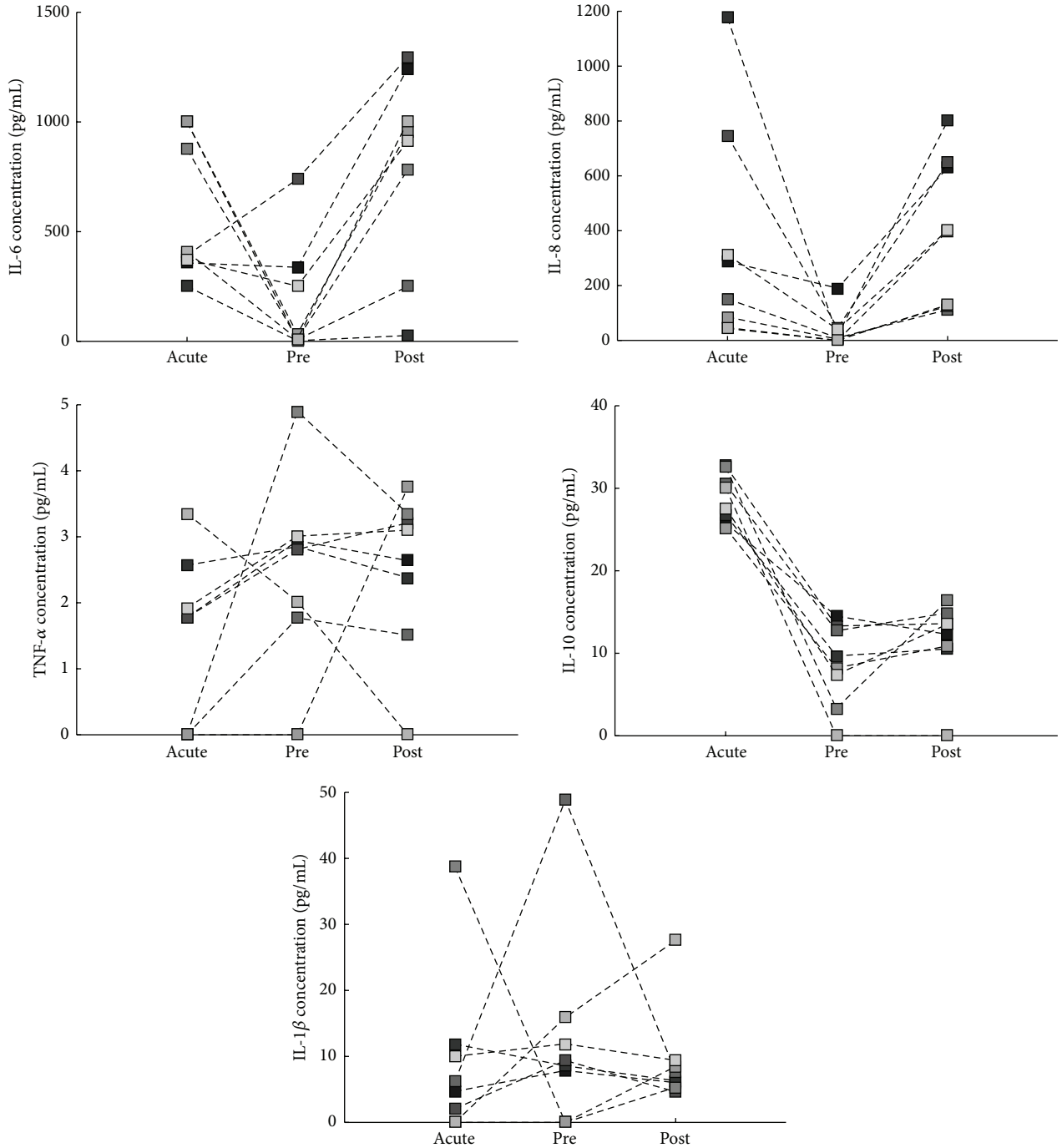

Figure 1: Cytokine levels in the synovial fluid of individual patients after ACL rupture. Squares represent values measured in single subjects ($n = 8$); each subject is represented with different grey values. Acute (less than 72 hours after ACL injury); pre (1 month after the trauma, in or before the arthroscopic ACL reconstruction surgery); post (1 month after the surgery).

from the acute ACL injury. The intra-articular stress that follows ACL reconstruction does not stimulate an increase in IL-10 capable of maintaining its levels in time higher than those measured in chronic ACL tear patients. Helmark and colleagues showed an increase in intra-articular IL-10 concentration secondary to specific exercise in patient with knee OA, suggesting an important dependence of the IL-10 production on muscular exercises [24]. Following 1 month of accelerated rehabilitation protocol, we also reported a slight trend towards higher concentrations of IL-10 postoperatively.

Cameron and colleagues observed that their patients could be divided into two subgroups when considering the synovial concentrations of IL-1β and TNF-α in ACL-deficient knees. One group presented levels of IL-1β and TNF-α that were high acutely after trauma and decreased to zero in time, whereas the other group had low levels of

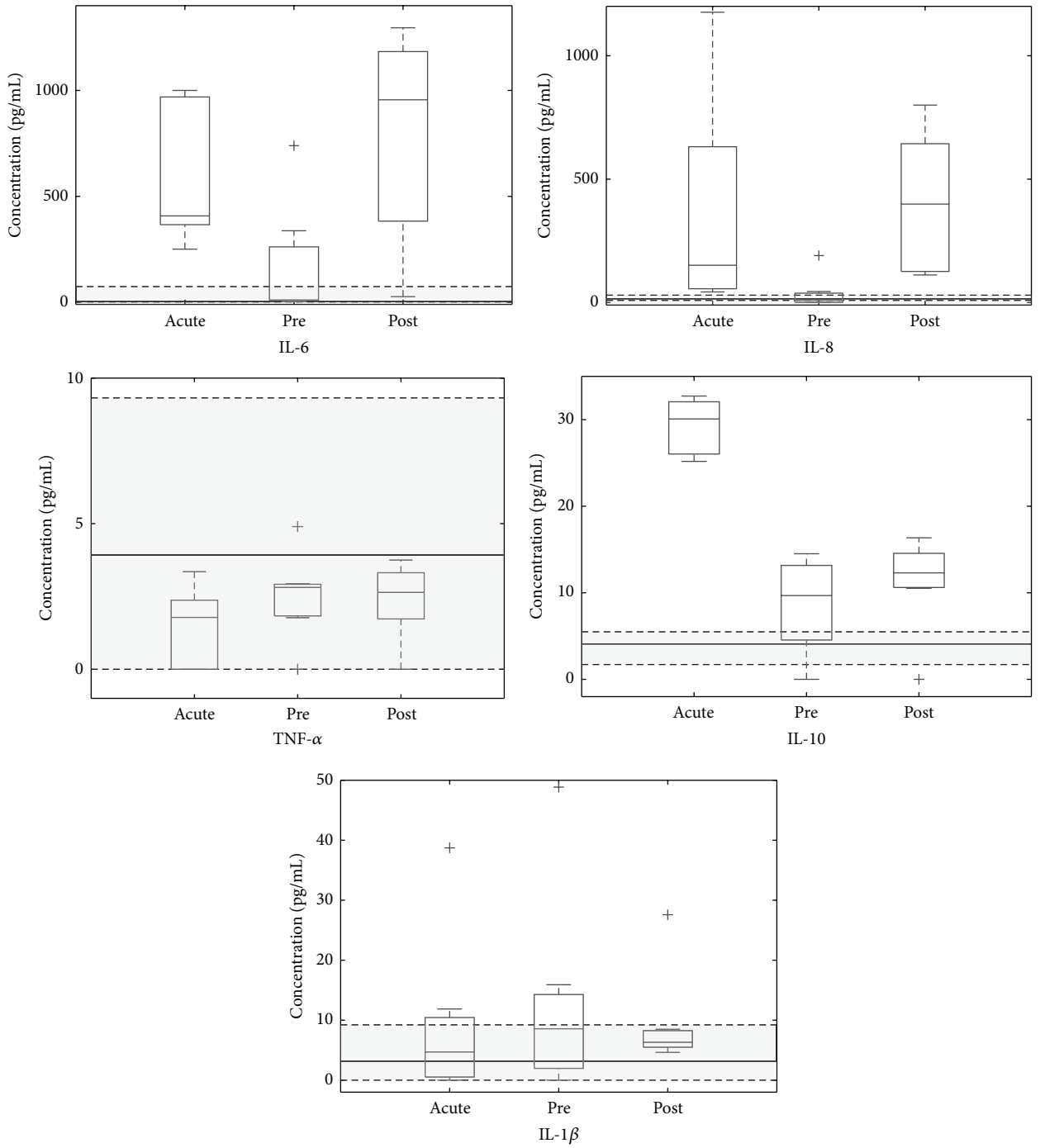

Figure 2: Time-related modifications of cytokine levels compared to chronic ACL group. Cytokine concentrations measured at different time points in the longitudinal group ($n = 8$) are represented in the box-plot. In each box-plot, the box is built within the third (upper bound) and first (lower bound) quartiles (i.e., Q_3, Q_1); the middle line represents the median. Whiskers represent data maximum (upper whisker) and minimum (lower whisker). + indicates data outliers, defined as data points below $Q_1 - 1.5 \times (Q_3 - Q_1)$ or above $Q_3 + 1.5 \times (Q_3 - Q_1)$. Grey shaded area represents the interquartile range of the chronic ACL tear group ($n = 17$).

these cytokines both acutely after trauma and four weeks postoperatively [25]. Based on these data, they speculated that on the basis of their IL-1β and TNF-α profiles patients could be at different risk of developing posttraumatic OA. However, data demonstrating that patients with high levels of IL-1β and TNF-α following acute ACL rupture have a higher incidence of OA compared to those presenting low levels of these inflammatory cytokines are still lacking. The data that we have obtained in the present study cannot support Cameron's hypothesis given the small size of the patient population. Even if IL-1β and TNF-α are two of the most studied and important cytokines in the pathophysiology

of OA, more studies to understand their specific role in ACL tear patients are required. Our data showed *acute* levels of IL-1β and TNF-α comparable to *presurgery* levels and *postsurgery* levels. Essentially, no differences can be reported between longitudinal ACL group and control group, in agreement with previous literature. Indeed, Zysk and coworkers investigated the synovial fluid concentrations of three proinflammatory cytokines (TNF-α, IL-1β, and IL-6) before ACL surgery (24±7 days after ACL rupture) and 7 days after the surgical operation. They reported that IL-6 levels increased significantly from the pre- to the postoperative measurements whereas IL-1β and TNF-α concentrations remained unchanged throughout the course of ACL surgery [18].

Interestingly, *in vitro* studies reported that cytokines as IL-1β and TNF-α have a role in stimulating the synthesis of other cytokines such as IL-6 and IL-8. However different cytokine trends in response to articular events underline the complexity of multiple cascades that could stimulate the synthesis and release of specific cytokines [23].

We are aware of some limitations of the results obtained in this study. We present the data obtained in a limited number of patients, but the prospective design of this pilot study, the strict criteria of samples collection, and storage and analysis and surgical management are very positive aspects. Moreover, we had no access to synovial samples from healthy knees for ethical reasons, and to obviate to this problem we choose to compare data with those of a group of patients with chronic ACL tears.

5. Conclusion

This pilot study is to our knowledge the first one describing the temporal evolution of synovial IL-6, IL-8, IL-1β, IL-10, and TNF-α concentrations from the time of ACL rupture to the postsurgical follow-up in the same patients. These data suggest that ACL injury and ACL surgery have a great impact on IL-6, IL-8, and IL-10 levels in the synovial fluid. TNF-α and IL-1β levels in synovial fluid followed a different temporal pattern, since they did not increase following acute ACL injury and remained unaltered in time despite the ACL reconstruction surgery.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Design of the study was performed by Marco Bigoni, Marco Turati, Diego Gaddi, Vittorio Locatelli, Robert J. Omeljaniuk, and Antonio Torsello. Marco Bigoni, Marco Turati, Alberto Castelnuovo, Massimiliano Piatti, Diego Gaddi, Daniele Munegato, Paola Sacerdote, Massimo Gorla, and Silvia Franchi performed the experiments. Marta Gandolla, Alessandra Pedrocchi, Marco Turati, Alberto Castelnuovo, Silvia Franchi, Daniele Munegato, Diego Gaddi, and Antonio Torsello analyzed the results. Drafting of the paper was done by Marco Bigoni, Marco Turati, Marta Gandolla, Massimiliano Piatti, Alberto Castelnuovo, Daniele Munegato, and Diego Gaddi. Paper final revision was performed by Paola Sacerdote, Robert J. Omeljaniuk, Vittorio Locatelli, Antonio Torsello, and Marta Gandolla. All authors approved the final version of the paper for submission.

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Review Article

The Role of Toll-Like Receptor 4 in Infectious and Noninfectious Inflammation

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Toll-like receptor 4 (TLR4) belongs to the family of pattern recognition receptors (PRRs). They are highly conserved receptors that recognize conserved pathogen-associated molecular patterns (PAMPs), thus representing the first line of defense against infections. TLR4 has been long recognized as the sensing receptor for gram-negative lipopolysaccharide (LPS). In addition, it also binds endogenous molecules produced as a result of tissue injury. Hence, TLR4 represents a key receptor on which both infectious and noninfectious stimuli converge to induce a proinflammatory response. TLR4-mediated inflammation, triggered by exogenous or endogenous ligands, is also involved in several acute and chronic diseases, having a pivotal role as amplifier of the inflammatory response. This review focuses on the research progress about the role of TLR4 activation in infectious and noninfectious (e.g., sterile) inflammation and the effects of TLR4 signaling in some pathological conditions.

1. Introduction

The first function described for TLR4 was the recognition of exogenous molecules from pathogens (pathogen-associated molecular pattern molecules (PAMPs)), in particular the molecules from gram-negative bacteria (e.g., LPS) [1]. Recently, it has been widely demonstrated that TLR4 is also involved in the recognition of endogenous molecules released by injured tissues and necrotic cells. These molecules, called damage-associated molecular pattern molecules (DAMPs), induce the activation of a strong proinflammatory response through interaction with TLR4 [2]. Generally, inflammation has a protective role. It is a complex and coordinated process followed by the induction of resolution pathways that restore tissue integrity and function. However, in some cases, excessive and/or poorly regulated inflammatory response can be harmful for the organism. In several diseases with microbial (gram-negative infections) or nonmicrobial etiology (ischemia/reperfusion injury and neurodegenerative and neurological diseases) there is an involvement of TLR4 activation that, under certain circumstances, can contribute to disease progression.

2. TLR4 Signaling

TLR4 is expressed on the cell surface on both hematopoietic and nonhematopoietic cells, including endothelial cells [3], cardiac myocytes [4], and cells of the central nervous system (CNS) [5]. TLR4 is composed of a 608-residue extracellular domain and a 187-residue intracellular domain that is involved in the intracellular signaling cascade [6]. It has been demonstrated that transfection of TLR4 alone is not enough for LPS recognition, and physical association of TLR4 with myeloid differentiation 2 (MD2) on the cell surface is required for ligand-induced activation [7–9]. MD2 lacks transmembrane and intracellular domains and noncovalently associates with the extracellular domain of TLR4 by interaction with LPS, forming the TLR4/MD2 receptor complex [10]. Detailed crystallographic data are reported elsewhere [11–13]. Other accessory molecules that enhance LPS sensing are LPS-binding protein (LBP) and CD14 that favor the transferring of LPS monomers to MD2 and TLR4 [14]. After LPS binding, a dimerization of two TLR4/MD2 complexes occurs, resulting in conformational changes of the TLR4 homodimer that induce the recruitment of adaptor

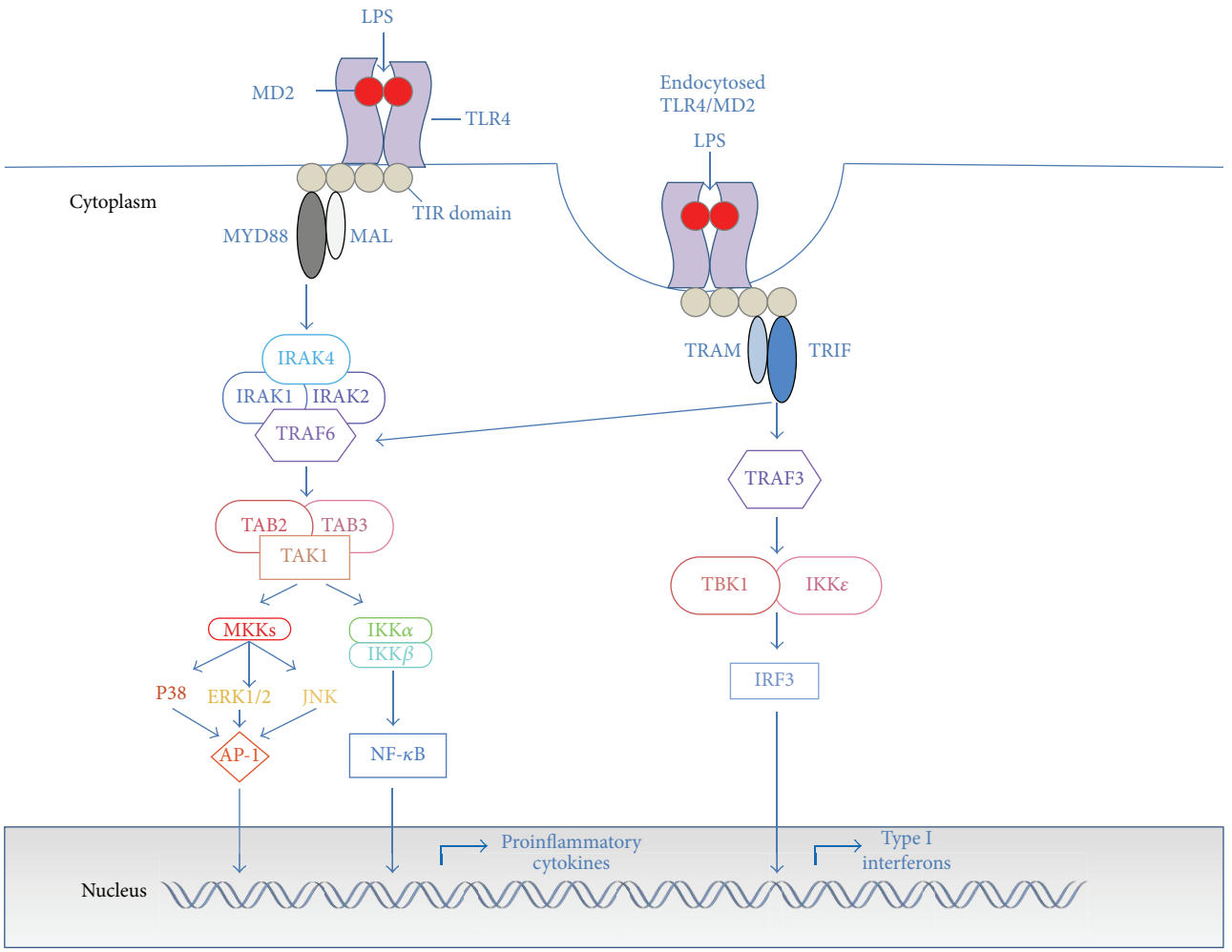

Figure 1: TLR4 intracellular signaling pathways. TLR signaling is triggered by ligand-induced dimerization of the receptors. TIR domains of TLR4 recruit TIR domain-containing adaptor proteins MyD88 and MAL (MyD88-dependent pathway) or TRIF and TRAM (MyD88-independent pathway). MyD88-dependent pathway involves the recruitment and the activation of IRAKs (IRAK1, IRAK2, and IRAK4) and TRAF6 that induce TAK1 activation. TAK1, in turn, leads to MAP kinase kinase- (MKK-) mediated activation of MAPKs (p38, JNK, and ERK1/2) and activation of IKK complex. MAPKs and IKK complex induce activation and translocation in the nucleus of transcription factors such as NF-κB and AP-1. MyD88-independent pathway involves TRIF and TRAM adaptor proteins and, via TRAF3, the recruitment of TBK1/IKKε, followed by the activation and translocation in the nucleus of the transcription factor IRF3. MyD88-dependent pathway induces production of proinflammatory cytokines, and MyD88-independent pathway induces the production of type I interferons.

proteins containing Toll/interleukin-1 receptor-like (TIR) domains. These adaptors associate with the TLR4 cluster through homophilic interactions between TIR domains in the cytoplasmic tail of TLR4 and those present on the adaptors. Four adaptor proteins, involved in two distinct intracellular signaling pathways, have been described: myeloid differentiation primary response protein 88 (MyD88), MyD88-adaptor-like (MAL) protein, also known as TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF), also known as TIR domain-containing adaptor molecule-1 (TICAM-1), and TRIF-related adaptor molecule (TRAM) [15]. MyD88-mediated signaling occurs mainly at the plasma membrane and involves a rapid recruitment of MyD88 and MAL proteins. Engagement of these adaptor molecules stimulates the recruitment and the activation by phosphorylation of IL-1R-associated kinases (IRAKs), the association of TNF-receptor-associated factor 6 (TRAF6), and the downstream activation of transforming growth factor β-activated kinase 1 (TAK1), mediated by the adaptor proteins, TAK1-binding protein 2 and TAK1-binding protein 3 (TAB2 and TAB3). TAK1 in turn activates the mitogen-activated protein kinases (MAPKs), JUN N-terminal kinase (JNK), p38, extracellular signal-regulated kinases (ERK1/2), and the IkB kinase complex (IKK), leading to the activation of important transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), that promote the production of proinflammatory cytokines [15] (Figure 1). Activation of MyD88-independent pathway occurs in the endosomal compartment after internalization of the TLR4-MD2 complex. It involves the recruitment

Table 1: TLR4 ligands.

TLR4 ligands	Activity	References
Exogenous natural ligands		
Lipopolysaccharides from gram-negative bacteria	Agonist	[20]
F protein of syncytial virus	Agonist	[21]
Mannuronic acid polymers from gram-negative bacteria	Agonist	[22]
Teichuronic acid from gram-positive bacteria	Agonist	[23]
Chlamydia pneumoniae HSP60	Agonist	[24]
Flavolipin from *Flavobacterium meningosepticum*	Agonist	[25]
Mannan from *S. cerevisiae* and *C. albicans*	Agonist	[26]
Dengue virus NS1 protein	Agonist	[27]
Plant paclitaxel	Agonist	[28]
Lipopolysaccharides from *Rhodobacter* sp.	Antagonist	[29]
Lipopolysaccharide-like (CyP) from *Oscillatoria* sp.	Antagonist	[30]
Endogenous ligands		
Extracellular matrix ligands		
Hyaluronan	Agonist	[31]
Biglycan	Agonist	[32]
Fibronectin	Agonist	[33]
Heparan sulphate	Agonist	[34]
Tenascin-C	Agonist	[35]
Intracellular and secreted endogenous ligands		
HMGB1	Agonist	[36]
HSP22	Agonist	[37]
HSP60	Agonist	[38]
HSP70	Agonist	[39]
HSP72	Agonist	[40]
HSP70L1	Agonist	[41]
HSP Gp96	Agonist	[42]
Calcineurin B	Agonist	[43]
β-defensin 2	Agonist	[44]
S100 proteins	Agonist	[45]
Surfactant protein A	Agonist	[46]
Resistin	Agonist	[47]
Fibrinogen	Agonist	[48]
Amyloid-β	Agonist	[49]

of adaptor proteins TRIF and TRAM, activation of TNF receptor-associated factor 3 (TRAF3), and the induction of IFN regulatory factor 3 (IRF3) nuclear translocation, mediated by tank-binding kinase 1 (TBK) and IKKε. IRF3 transcription factor promotes the production of type I IFNs (Figure 1). CD14 favors receptor complex internalization [14], even though also CD14-independent translocation of the receptor complex to the endosome and TRIF signaling has been recently demonstrated [16].

Beyond the induction at the transcriptional level of proinflammatory mediators, TLR4 interaction with LPS also orchestrates the induction of mediators such as microRNAs (miRNAs) that posttranscriptionally regulate the shutdown of the proinflammatory response and induce a state of temporary refractoriness to further LPS stimulation. Therefore, a tight regulation of TLR4 signaling is important in tissue homeostasis to avoid excessive inflammation and to induce tissue repair following infection or injury [17, 18].

3. TLR4 Ligands

Since the discovery of the role of TLR4 as a sensor of bacterial LPS by Poltorak et al. [19] and Qureshi et al. [20], several other ligands have been identified from both exogenous sources from both the host tissues and cells (Table 1).

Exogenous ligands (PAMPs) are molecules isolated from bacteria, viruses, fungi, plants, and cyanobacteria. Most are agonists of TLR4/MD2 complex [19–28]. However, from bacteria and cyanobacteria (*Rhodobacter* and *Oscillatoria* species, resp.), TLR4 antagonists have also been obtained and their mechanism of action has been well characterized [29, 30]. These antagonists were employed both *in vitro* and

in vivo in animal models of diseases, allowing investigation of the effects of TLR4 signaling modulation [29, 30].

Endogenous ligands (DAMPs) belong to two main groups: (a) molecules originated from extracellular matrix and [31–35] (b) intracellular mediators passively released or actively secreted by cells [36–49]. Even though for some DAMPs the ability to activate TLR4-mediated signaling has been questioned [50] and the mechanisms of interaction with TLR4/MD2 have not been investigated yet (no crystallographic data are available), there is no doubt that some endogenous molecules could use TLR4 to induce a proinflammatory response. A prototypic molecule of the extracellular matrix that can induce TLR4-mediated inflammation is the glycosaminoglycan hyaluronan. In normal conditions, hyaluronan is present in tissues in a high molecular form (up to 10^6 Da). After tissue injury, it is degraded into small fragments, which have been shown to activate macrophages via TLR4 both *in vitro* and *in vivo* [31, 51]. Endogenous intracellular triggers of TLR4 include the DNA-binding protein high-mobility group box 1 (HMGB1) and cellular heat shock proteins (HSPs). After cell damage and necrosis, these molecules are released in the extracellular milieu, thus inducing a strong proinflammatory response mediated by TLR4 [36, 40, 52]. Beyond its role in sterile inflammation, HMGB1 is also actively released by immunocompetent cells after exposure to the products of pathogenic bacteria, thus representing a common mediator at the intersection of infectious and noninfectious inflammatory response [36]. Only for HMGB1, among endogenous TLR4 ligands, more detailed studies using surface plasmon resonance were done to confirm the specific binding of HMGB1 to TLR4 [53].

4. The Role of TLR4 in Infectious Diseases

The central role played by TLR4 in gram-negative infections comes from studies on TLR4-mutated or TLR4-deficient mice [54]. It has been observed that TLR4-mutated strain C3H/HeJ is hyporesponsive to LPS and highly susceptible to infection by gram-negative bacteria such as *Salmonella typhimurium* and *Neisseria meningitidis* [54–56]. On the other hand, studies demonstrated that $TLR4^{-/-}$ mice were protected from endotoxin shock induced by *E. coli*, thus supporting TLR4 as a possible target for therapeutic intervention in sepsis [57]. In humans, genetic studies on TLR4 polymorphisms (missense mutations D299G and T399I) gave conflicting results. Some studies have linked TLR4 polymorphisms to an increased susceptibility to sepsis due to gram-negative infection; other studies failed to confirm this (reviewed in [58]). Furthermore, results with primary cells isolated from individuals according to D299G/T399I haplotypes did not show any difference in LPS responsiveness [59]. More recent studies have shown a single nucleotide polymorphism rs11536889 in $3'$-untranslated region of TLR4 associating with periodontitis [60] and organ failure in sepsis [61]. This polymorphism was shown to affect expression of TLR4 on the cell surface and IL8 production in response to LPS possibly through miRNA regulation [62].

Given the role of TLR4 in the activation of the proinflammatory response during infection, pharmacological approaches targeting TLR4 have been developed with the aim to control host's deleterious proinflammatory response called "cytokine storm" occurring in the early phase of sepsis. Unfortunately, the results of clinical trials with molecules targeting TLR4 were disappointing [63], suggesting that immune suppression, which follows the "cytokine storm," represents the leading process in the progression of sepsis [64].

5. The Role of TLR4 in Noninfectious Diseases

5.1. Ischemia/Reperfusion (I/R) Injury. Tissue I/R injury is caused by a sudden interruption of the blood supply to an organ followed by its restoration. Hypoxia induces cell injury and tissue damage with the release of several DAMPs, including HMGB1 [65]. Reperfusion is essential to preserve the organ; however, the exposure of the ischemic area to restored blood flow can lead to an acute inflammatory response causing an additional extensive tissue destruction, a phenomenon termed "reperfusion injury." DAMPs released by necrotic and distressed cells, through interaction with PRRs, induce the release of proinflammatory mediators by resident macrophages and dendritic cells that recruit, in the reperfusion phase, neutrophils, monocyte, and lymphocytes from blood flow to the ischemic organ. Indeed, the recruitment and activation of neutrophils produce the release of reactive oxygen and nitrogen species and of proteolytic enzymes that are highly cytotoxic and exacerbate tissue damage [66]. Acute myocardial and cerebral infarctions, as well as solid organ transplantations, are all conditions in which I/R injury occurs. In myocardial infarction, reperfusion injury accounts for up to 50% of myocardial infarct size, thus reducing the beneficial effects of reperfusion [67]. In organ transplantation, I/R injury resulting from cold preservation and warm reperfusion of the transplanted graft directly contributes to allograft rejection [68]. Several studies in animal models clearly demonstrated that TLR4, being the target of several DAMPs, plays a key role in I/R injury [69–74]. A great deal of information about the role of TLR4 in I/R injury came from studies using TLR4-mutated (C3H/HeJ) or $TLR4^{-/-}$ mice. In experimental models of acute myocardial infarction, TLR4 mutant mice showed a reduction of infarct size, decreased activation of NF-κB and AP-1, and lower myocardial mRNA levels of interleukin-1β (IL-1β), monocyte chemotactic factor-1 (MCP-1), and interleukin-6 (IL-6), in comparison with wild-type mice [75]. In $TLR4^{-/-}$ mice we observed reduced infarct sizes and polymorphonuclear cells infiltrating the ischemic area (unpublished observations). The importance of TLR4 was also highlighted by studies of cold I/R injury in syngeneic heart transplant between TLR4 mutant and wild-type mice. The results demonstrated that TLR4 signaling on both donor and recipient cells contributes to systemic and intragraft inflammatory response [76]. Interesting results have also been obtained using a synthetic inhibitor of TLR4/MD2 complex (Eritoran) and a natural cyanobacterial TLR4 antagonist in a murine model of myocardial I/R injury. The results suggest that the

downregulation of TLR4-induced proinflammatory response has beneficial effects in reducing tissue damage [77, 78] and this was associated, in the experiments with the cyanobacterial antagonist, with a reduced number of polymorphonuclear leukocytes infiltrating the ischemic area [78]. Confirming results about the positive effects of TLR4 targeting in I/R injury were also obtained in experimental models of hepatic I/R injury in which decreased inflammatory mediators and inhibition of HMGB1 release from hepatocytes were observed after treatment with Eritoran [79]. Indeed, no data are available about the impact of TLR4 signaling inhibition in I/R injury on the long-term tissue repair. Our preliminary data (unpublished results) in a mouse model of acute myocardial infarction using the cyanobacterial TLR4 antagonist suggest that early inhibition of TLR4 signaling just before reperfusion positively affects tissue remodeling, since long-term cardiac function was better in mice treated with the antagonist in comparison to mice treated with vehicle. However, further in-depth studies are needed to clarify the role of TLR4 signaling in tissue repair.

5.2. Neurodegenerative and Neurological Diseases. Neuroinflammation is the common hallmark of several neurodegenerative and neurological diseases [80–82]. In the CNS, microglial cells are resident phagocytes that constantly control the extracellular environment and sense for the presence of pathogens or injured cells. Microglial cells are the immunological "sentinels" of the CNS: they express TLR4 and are highly responsive to LPS *in vitro* [83]. Microglial activation by noxious stimuli represents a defensive response with the aim to restore tissue homeostasis. In several pathological conditions, however, persistent exposure to danger signals can cause aberrant microglia activation with the production of proinflammatory mediators and the release of reactive oxygen and nitrogen species that result in neuronal dysfunction and/or neuronal cell loss. It has been reported that direct TLR4 stimulation with LPS produces immediate and long-term memory deficits in mice models of endotoxemia, especially in aged mice [84, 85]. In similar experiments, the contribution of HMGB1 on memory impairment mediated by both TLR4 and receptor for advanced glycation end products (RAGE) has been demonstrated [85, 86]. Increased expression of TLR4 in microglial cells has been observed in animal models and patients of Alzheimer's disease (AD) [87–89], Parkinson's disease (PD) [90], and Amyotrophic Lateral Sclerosis (ALS) [91, 92].

In AD, amyloid-β (Aβ) oligomers directly induce microglial activation through several receptors, including TLR4 [49, 80]. Activated microglia has an active role not only in the production of proinflammatory mediators but also in the phagocytosis of Aβ. Indeed, the continuous formation of Aβ caused, at least in part, by positive feedback between inflammation and amyloid precursor protein processing drives a chronic detrimental and nonresolving proinflammatory loop. In this contest, the role of TLR4 is not clear, probably due to the complex mechanisms controlling reacting microglia phenotypes [80, 93]. *In vitro* studies showed a role of TLR4 in the Aβ-induced neurotoxicity [87, 88]. Differently, some experimental studies using transgenic AD mice carrying mutated TLR4 showed reduced microglial activation but also reduced Aβ clearance with exacerbation of cognitive deficits [94]. These data suggest that there is a delicate balance between the production of proinflammatory mediators and Aβ phagocytosis in glial cells, and an inefficient clearance of Aβ, only partially TLR4-dependent, could be involved in disease progression [80, 93, 95, 96]. In support of this hypothesis, Michaud et al. [97] recently demonstrated that treatment of transgenic AD mice with monophosphoryl lipid A (MPLA), a LPS-derived analog of gram-negative lipid A, led to a significant reduction of Aβ accumulation in the brain and enhanced cognitive function. MPLA is a TLR4 agonist but does not induce large amounts of proinflammatory mediators. In this context, MPLA was shown to induce a potent phagocytic response by microglia while triggering a moderate inflammatory response *in vivo*.

In PD, where accumulation of extracellular misfolded α-synuclein is observed, contrasting results about the role of TLR4 have been obtained. In a mouse model of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), TLR4 was shown to mediate cell death of dopaminergic neurons and TLR4-deficient mice were partially protected against MPTP toxicity [98]. Indeed, experimental TLR4 deficiency was shown to decrease α-synuclein clearance *in vitro* by murine microglia [99, 100].

The studies about the role of TLR4 in other neurodegenerative and neurological diseases, ALS and epilepsy, gave more clear results. In ALS upregulation of TLR4 and cytoplasmic HMGB1 were observed in reactive glia (astrocytes and microglia) and neurons of the spinal cord in ALS patients [91]. In a mouse genetic model of ALS (superoxide dismutase 1-mutant mice), chronic administration of LPS (once every 2 weeks for 3 months) in presymptomatic mice accelerated motor neuron degeneration and disease progression [101]. Furthermore, De Paola et al. [92] demonstrated that the chronic treatment with a TLR4 antagonist in a mouse model of spontaneous motor neuron degeneration (wobbler mice) produced potent anti-inflammatory effects (reduction of gliosis and TNF-α production) with significant improvements of motor functional tests. In epilepsy, analyses of hippocampal specimens obtained at surgery from patients with drug-resistant temporal lobe epilepsy showed increased TLR4 and HMGB1 expression in glial cells (astrocytes) and neurons [102]. Moreover, in acute and chronic mice models of seizures a proconvulsant pathway involving TLR4-HMGB1 axis was demonstrated. Intriguingly, antagonists targeting TLR4 were shown to delay seizure onset and decrease acute and chronic seizure recurrence [102].

6. Conclusions

Since its discovery, a great deal of experimental data supported TLR4 as a key player of the inflammatory process due to both infectious and noninfectious stimuli. In several pathological conditions TLR4 engagement contributes to disease resolution; however, when TLR4 activation pathways are poorly regulated, it can contribute to disease progression. More information is needed to clarify the role of TLR4 engagement in the different phases of I/R injury or during the

neurodegenerative processes, with a particular attention to the effects of TLR4 signaling on the fine phenotypic changes occurring *in vivo* in both peripheral (macrophages) and CNS (microglia) innate immune cells. In this framework, TLR4 targeting could represent a successful means to manipulate macrophages and glial cells activation and the development of molecules acting on TLR4 could represent new disease-modifying therapeutic agents for the treatment of I/R injury or for neurodegenerative diseases.

Competing Interests

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

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Research Article

Effect of High, Medium, and Low Molecular Weight Hyaluronan on Inflammation and Oxidative Stress in an *In Vitro* Model of Human Nasal Epithelial Cells

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IL-17A is involved in the activation of oxidative stress and inflammation in nasal epithelial cells. Hyaluronan (HA) in its high molecular weight form (HMW-HA) shows anti-inflammatory responses in contrast to low and medium molecular weight HA (LMW-HA and MMW-HA). The aim of this study was to investigate the pro- or anti-inflammatory biologic function of HA at different molecular weight in an *in vitro* model of nasal inflammation IL-17A mediated. We evaluated the ERK1/2 and IκBα phosphorylation, NF-κB signal pathway activation, ROS production, IL-8 and NOX-4 protein, and mRNA levels, in nasal epithelial cells RPMI 2650 stimulated with recombinant human (rh) IL-17A. Furthermore, the cells were treated with HMW-HA, MMW-HA, LMW-HA, and U0126. Our results showed that rhIL-17A increased the ERK1/2, IκBα phosphorylation and NF-κB signal pathway activation, ROS production, IL-8 and NOX-4 proteins, and mRNA levels. The addiction of HMW-HA or U0126 showed a significant downregulatory effect on inflammation due to the rhIL-17A stimulation in nasal epithelial cells. IL-17A is able to generate oxidative stress and inflammation via the activation of ERK1/2/NF-κB pathway in nasal epithelial cells. The HMW-HA might represent a coadjuvant of the classic anti-inflammatory/antioxidative treatment of nasal epithelial cells during IL-17A nasal inflammation.

1. Introduction

Allergic rhinitis is an extremely common medical problem characterized by nasal congestion, clear rhinorrhea, sneezing, and itching. The presence of an uncontrolled inflammation in the upper airways may compromise the control of allergic rhinitis with a consequent progression of the diseases [1]. IL-17A and oxidative stress are involved in the development and progression of allergic rhinitis by the activation of nasal epithelial cells [2].

Hyaluronan (HA) is a glycosaminoglycan widely distributed in tissues and is a normal constituent of airway secretions [3, 4]. It is a major component of the extracellular matrix and it plays a key role in regulating inflammation that is associated with accumulation and turnover of HA polymers by multiple cell types. Increasingly through the years, HA has become recognized as an active participant in inflammatory, angiogenic, fibrotic, and cancer promoting processes [5].

In the airways, HA is produced by submucosal glands and by superficial airway epithelial cells [6, 7]. It is synthesized by hyaluronan synthases (HAS) at the plasma membrane and normally released, as a high molecular weight polymer (HMW) (>1000 kDa), into the extracellular milieu [8, 9]. Low molecular weight HA (LMW, defined as <500 kDa) can be produced by the breakdown of HMW-HA to LMW-HA, or by *de novo* synthesis by hyaluronan synthases during inflammatory process [10, 11]. The differential activities of HA

and its degradation products are due, in part, to regulation of multiple HA-binding proteins, including cluster of differentiation 44 (CD44) [10].

Most HA functions have been shown to be size-dependent: the HMW molecules have been reported to exert anti-inflammatory and immunosuppressive effects, while LMW stimulate gene expression and synthesis of proinflammatory protein such as cytokines and chemokines [10, 12]. These results strongly support the role of HA and HA-binding proteins in lung pathobiology of asthma [13, 14]. HA appears in low concentrations in bronchoalveolar lavage fluid (BAL) from healthy individuals and is elevated in BAL of asthma patients [15, 16]. The concentration of HA in BAL was found to significantly correlate with the severity of asthma [17]. However, the role of HA homeostasis in human asthma and allergic rhinitis has not been thoroughly explored. *In vitro* studies showed that LMW-HA induces, via ERK1/2 and NF-κB signaling, the production of IL-8 in transformed bronchial epithelial cells [18], suggesting that LMW-HA is able to play a role in acute lung inflammation. However, there were no studies showing the role of LMW-HA in nasal inflammation during allergic rhinitis.

HA is an endogenous compound having an important role in mucociliary clearance and mucosal surface healing and repair of nasal epithelial cells [19]. It was observed that the addition of sodium HA to intranasal corticosteroid and systemic antihistamine reduced the neutrophil count seen on nasal cytology in patients with allergic and nonallergic rhinitis and improved several clinical and endoscopic parameters while being well tolerated. Furthermore, the use of intranasal sodium HA in patients undergoing functional endoscopic sinus surgery for nasal polyposis augmented the improvement in mucociliary clearance observed following this procedure and improved several clinical and endoscopic parameters [19, 20]. These data provide encouraging evidence of the beneficial effects of sodium HA in the care of patients with altered function of nasal epithelial cells and suggest its potential involvement in the control of nasal inflammation and oxidative stress.

We aimed to perform a study to test the use of LMW-HA (500 kDa), MMW-HA (~900 kDa), and HMW-HA (~1600 kDa) in an *in vitro* model of oxidative stress (ROS production and NOX-4 expression) and inflammation (IL-8 synthesis) generated by rhIL-17A in nasal epithelial cells. This study might be appropriate to identify the potential therapeutic application of HMW-HA as coadjuvant of the classic anti-inflammatory treatment in the pathological inflammation and oxidative stress generated in nasal epithelium during the chronic inflammation of the airways.

2. Materials and Methods

2.1. Nasal Epithelial Cell Cultures. RPMI 2650 cell lines (ATCC-CCL-30) were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) and supplied at Passage 26. This line represents an appropriate *in vitro* nasal model able to grow a polarized epithelium resembling nasal mucosa [21]. Cells were cultured in complete culture medium (MEM minimum essential media containing 10% FCS, L-glutamine 2 mM, gentamicin 50 mg/mL, MEM NEAA 0.5%, and sodium pyruvate 1 nM).

2.2. Stimulation of RPMI 2650 Cells. The cells were seeded in standard six-well culture plates in MEM 10% FCS and grown to 60–70% confluence prior to treatment. RPMI 2650 cells were stimulated with recombinant human IL-17A (rhIL-17A) (R&D Systems, Minneapolis, MN) (20 ng/mL) as previously described [2].

To determine the role of the MAPK pathways in the activation of oxidative stress and IL-8 production, RPMI 2650 cells were stimulated with rhIL-17A for 30 min, 6 hrs, or 18 hrs in the presence or absence of inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto) butadiene monoethanolate) (25 μM) (Sigma-Aldrich s.r.l., Milan, Italy) (specific inhibitor of MEK1 and MEK2 MAP kinase kinase; MAPKK). The RPMI 2650 cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) as positive control for ERK1/2 (extracellular-signal-regulated kinases) and IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) activation.

To test the activity of different HA molecular weight on the oxidative stress and IL-8 production, RPMI 2650 cells were stimulated with rhIL-17A for 30 min, 6 hrs, or 18 hrs in the presence or absence of HMW-HA (1600 kD, IALUCLENNY, 0.12%, Chiesi Farmaceutici S.p.A.) (100 μg/mL) or MMW-HA (900 kDa, YABRO, 0.3%, Ibsa Farmaceutici Italia) (100 μg/mL) or LMW-HA (370 kDa, cod. H7630, Sigma Chemical Co. St. Louis, MO) (100 μg/mL). To confirm the specific action of HA, HMW-HA samples were degraded with *Streptomyces* hyaluronidase (HAdase, catalyzing the random hydrolysis of HA) (0.05 Units/2 μg) (Sigma Chemical Co., St. Louis, MO) at 60°C for 72 hours followed by 10 minutes at 65°C.

2.3. ERK1/2, pIκBα, and NF-κB Activation. The effect of rhIL-17A on ERK1/2 and IκBα activation was evaluated in RPMI 2650 cells stimulated for 30 minutes. We performed western blot analysis in total cell lysates and nuclear extracts. In total extract, we studied pERK1/2 using an anti-phospho ERK1/2 rabbit monoclonal antibody, pIκBα using an anti-phospho IκBα rabbit antibody (Cell Signaling Technology, Beverly, MA), and anti-β-actin (Sigma St. Louis, MO). In nuclear extracts, we studied the nuclear translocation of NF-κB, using an anti NF-κB p65 (C-20) antibody (Santa Cruz Biotechnology, Inc., MI, Italy). Nuclear extracts were obtained by NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific), providing an efficient cell lysis and separate cytoplasmic and nuclear protein fractions by centrifugation. Additionally, we evaluated ERK1/2 and NF-κB activation using two commercially available ELISA kits (SuperArray Bioscience, Frederick, MD) that measure phosphorylated and total ERK1/2, as well as phosphorylated and total NF-κB. Results are expressed, respectively, as pERK1/2/tERK1/2 ratio and pNF-κB/tNF-κB ratio and normalized to protein content.

2.4. Detection of Intracellular ROS. The intracellular reactive oxygen species (ROS) generation was evaluated in RPMI

2650 stimulated with rhIL-17A (20 ng/mL) for 6 hrs. The cells were trypsinised, washed in PBS, collected in FACS tubes, and then incubated with 1 mM of the oxidant-sensitive dye 2,7-dichlorofluorescein diacetate (DCFH-DA) in PBS for 10 min in the dark at room temperature. After washing, cells were suspended in PBS and then analysed by flow cytometry for fluorescence positive cells using a FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA, USA). Negative controls consisted of RPMI 2650 cells cultured without DCFH-DA. Gating on the cells, excluding debris, was performed using forwards and sideways scatter patterns.

2.5. Detection of NOX-4 and IL-8 by Western Blot. NOX-4 (NADPH oxidase) and IL-8 were evaluated in RPMI 2650 stimulated with rhIL-17A for 18 hrs in the presence or absence of HAs. Total proteins were extracted from stimulated RPMI 2650 cells using a lysis buffer (NaCl 50 mM, Tris-HCl 10 mM, EDTA 5 mM, and NP-40 1%) containing protease and phosphatase inhibitors. Protein concentration was assessed using the Bradford method. The total protein extracts were separated by SDS-PAGE on 10% gradient gels followed by electroblotting onto nitrocellulose membranes. Western blot was performed using a primary rabbit polyclonal anti-NOX-4 (H-330, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a mouse monoclonal anti-IL-8 (B-2, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). β-actin (Sigma-Aldrich) was used as a housekeeping protein to control the total amount of protein in each sample. Primary antisera were visualized with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) and developed with an enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, UK). Approximate molecular masses were determined using calibrated prestained standards (GE Healthcare).

2.6. Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of NOX-4 and IL-8. Total RNA was extracted from RPMI 2650 cells with TRIzol Reagent (Invitrogen) following the manufacturer's instructions and was reverse-transcribed into cDNA, using M-MLV-RT and oligo $(dT)_{12\text{-}18}$ primer (Invitrogen). Quantitative real-time PCR of NOX-4 and IL-8 transcripts was carried out on StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (prevalidated TaqMan Gene Expression Assay for NOX-4, Hs00418356m1, and IL-8, Hs00174103m1; Assays on Demand, Applied Biosystems). NOX-4 and IL-8 gene expression were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control gene. Relative quantitation of gene expression was carried out with the comparative C_T method ($2^{-\Delta\Delta Ct}$) and was plotted as fold change compared to untreated cells chosen as the reference sample.

2.7. Gel Image Evaluation. Gel images were taken with an EPSON GT-6000 scanner and then imported into a National Institutes of Health Image analysis 1.61 program to determine band intensities. Data are expressed as arbitrary densitometric units corrected against the density of β-actin bands.

2.8. Statistics. We tested normal distribution of the data with Kolmogorov–Smirnov test. Analysis of variance (ANOVA) corrected with Fisher's test and t-test were used for comparisons. Data are expressed as mean ± standard deviation (SD). $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. ERK1/2 and IκBα Activation in RPMI 2650 Stimulated with rhIL-17A. The stimulation of RPMI 2650 with rhIL-17A for 30 minutes significantly increased pERK1/2 and pIκBα activation in comparison to untreated cells ($p < 0.0001$) by western blot analysis. The preincubation of the cells with U0126 (25 μM) significantly decreased the levels of pERK1/2 and pIκBα, as observed in the cells stimulated with rhIL-17A ($p < 0.0001$) (Figure 1(a)). Accordingly, the levels of pERK1/2/total ERK1/2 ratio and pNF-κB/total NF-κB ratio significantly increased in the cells stimulated with rhIL-17A ($p < 0.002$ and $p < 0.0001$, resp.) and showed a statistically significant decrease when the cells were preincubated with U0126 ($p < 0.003$ and $p < 0.002$ resp.) (Figure 1(b)).

3.2. ROS Production, NOX-4, and IL-8 Proteins in RPMI 2650 Stimulated with rhIL-17A. The ROS production showed a significant increase in RPMI 2650 cells stimulated for 6 hrs with rhIL-17A (20 ng/mL) ($p < 0.001$), compared to untreated cells. The pretreatment of the cells with U0126 (25 μM) significantly decreased ROS production in RPMI 2650 cells stimulated with rhIL-17A ($p < 0.02$) (Figure 2(a)).

NOX-4 and IL-8 protein significantly increased in RPMI 2650 cells stimulated for 18 hrs with rhIL-17A (20 ng/mL) ($p < 0.0001$ and $p < 0.0002$), in comparison to untreated cells. The preincubation of RPMI 2650 cells with U0126 (25 μM) significantly decreased NOX-4 ($p < 0.0005$) and IL-8 production ($p < 0.002$) in nasal epithelial cells stimulated with rhIL-17A (Figure 2(b)).

3.3. Effect of HMW-HA, MMW-HA, and LMW-HA on ERK1/2 and NF-κB Activation. The pretreatment of the RPMI 2650 with HMW-HA significantly inhibited the levels of pERK1/2 ($p < 0.0001$) and pIκBα ($p < 0.0001$) in the cells stimulated with rhIL-17A, compared to the cells treated with rhIL-17A alone (Figure 3(a)). Accordingly, the pretreatment of the RPMI 2650 with HMW-HA significantly reduced the pERK/total ERK ratio and pNF-κB/total NF-κB ratio in the cells stimulated with rhIL-17A compared to the cells treated with rhIL-17A alone ($p < 0.002$, $p < 0.02$, resp.) (Figure 3(b)). Conversely, the pretreatment of the RPMI 2650 with MMW-HA and LMW-HA did not control the activity of rhIL-17A on ERK1/2 and NF-κB pathway activation.

3.4. Effect of HMW-HA, MMW-HA, and LMW-HA on ROS Production, NOX-4, and IL-8 Synthesis. The pretreatment of RPMI 2650 with HMW-HA and MMW-HA reduced the induction of ROS production in the cells stimulated with rhIL-17A compared to the cells treated with rhIL-17A alone ($p < 0.002$ and $p < 0.03$, resp.). Conversely, the pretreatment of the cells with LMW-HA did not affect the ROS

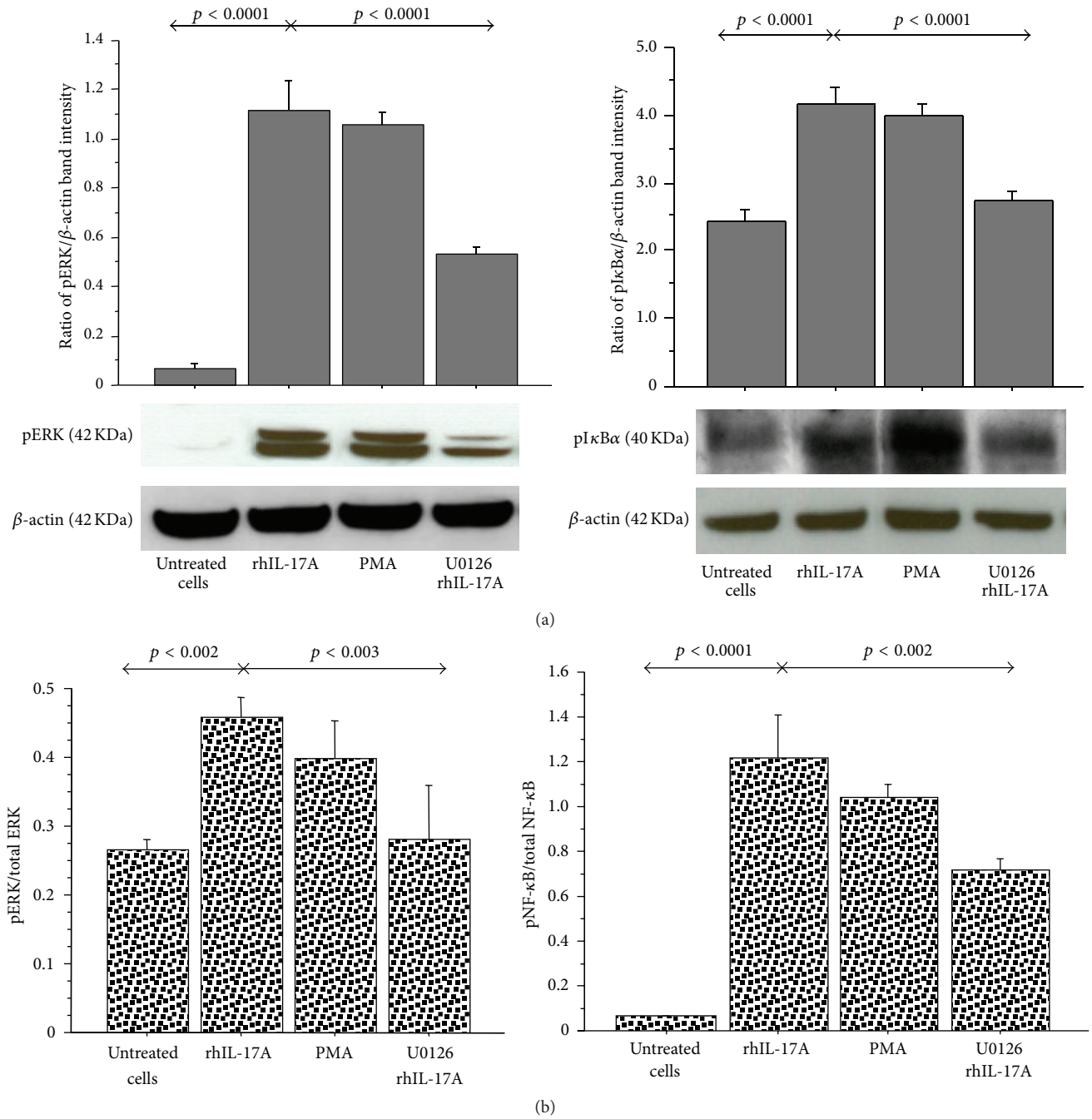

Figure 1: Effect of U0126 inhibitor on ERK and IκBα phosphorylation in RPMI 2650 cells stimulated with rhIL-17A. The cells were stimulated with rhIL-17A (20 ng/mL) or PMA (50 ng/mL) for 30 min in absence or presence of U0126 (25 μM). (a) pERK and pIκBα protein expression were evaluated in the cell lysates by western blot. The results were expressed as ratio of band intensity and β-actin of 3 separate experiments. Representative gel images of pERK, pIκBα, and β-actin are shown. (b) The activation of ERK1/2 and NF-κB for each experimental condition was tested for the pERK1/2/total ERK1/2 ratio and for the pNF-κB/total NF-κB, respectively, by ELISA and normalized for protein content. ANOVA with Fisher's test correction was used for the analysis of the data. $p < 0.05$ was statistically significant.

production generated by rhIL-17A stimulation in RPMI 2650 (Figure 4(a)).

The pretreatment of RPMI 2650 cells with HMW-HA significantly inhibited NOX-4 and IL-8 synthesis ($p < 0.0009$, $p < 0.0001$, resp.) in the cells stimulated with rhIL-17A compared to the cells treated with rhIL-17A alone (Figure 4(b)). Conversely, the pretreatment of the RPMI 2650 with MMW-HA and LMW-HA did not control the activity of rhIL-17A on NOX-4 and IL-8 synthesis.

3.5. Effect of U0126, HMW-HA, MMW-HA, and LMW-HA on Nuclear NF-κB Translocation. The stimulation of RPMI

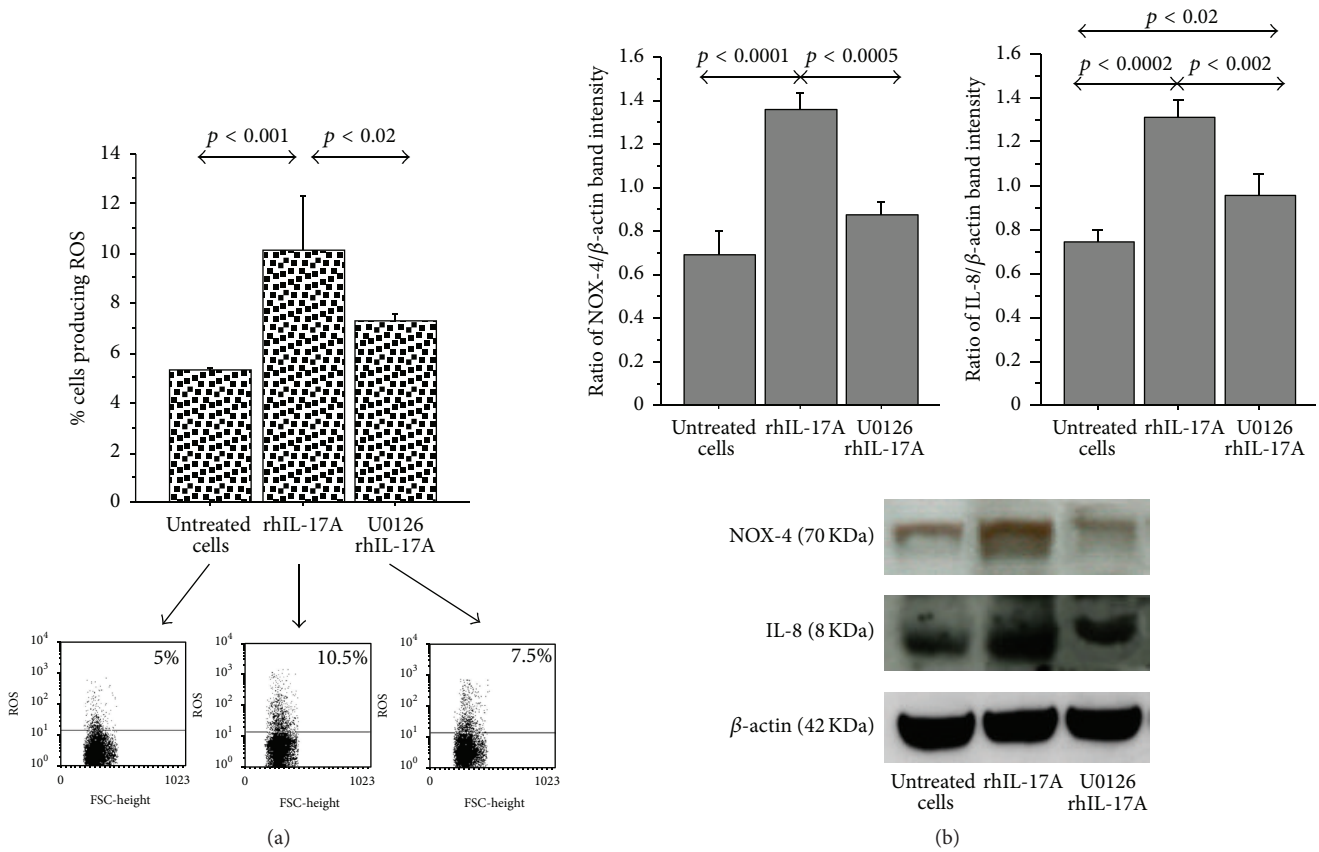

Figure 2: Effect of U0126 inhibitor in RPMI 2650 cells stimulated with rhIL-17A. (a) The cells were stimulated with rhIL-17A (20 ng/mL) for 6 hrs in absence or presence of U0126 (25 μM). ROS production was evaluated in the cells by flow cytometry. The bars represent the mean ± SD of 3 separate experiments. Representative flow cytometry are shown; (b) the cells were stimulated with rhIL-17A (20 ng/mL) for 18 hrs in absence or presence of U0126 (25 μM). NOX-4 and IL-8 protein expression were evaluated in the cell lysates by western blot. The results were expressed as ratio of band intensity and β-actin of 3 separate experiments. Representative western blot is shown. ANOVA with Fisher's test correction was used for the analysis of the data. $p < 0.05$ was statistically significant.

2650 with rhIL-17A for 30 minutes significantly increased the nuclear translocation of NF-κB in comparison to untreated cells ($p < 0.0001$) that was significantly decreased when the cells were preincubated with U0126 (25 μM) ($p < 0.0001$) (Figure 5(a)). The pretreatment of RPMI 2650 cells with HMW-HA significantly inhibited the nuclear levels of NF-κB ($p < 0.0001$) in the cells stimulated with rhIL-17A compared to the cells treated with rhIL-17A alone. Conversely, the pretreatment of the RPMI 2650 with MMW-HA and LMW-HA did not control the activity of rhIL-17A on nuclear translocation of NF-κB (Figure 5(b)).

3.6. Effect of U0126, HMW-HA, MMW-HA, and LMW-HA on NOX-4 and IL-8 mRNA. Despite the data on the expression of NOX-4 protein, we did not observe modification of NOX-4 mRNA transcript in the cells stimulated with rhIL-17A for 2, 6, and 18 hrs in the presence or absence of HAs (data not shown). The stimulation of RPMI 2650 with rhIL-17A for 18 hrs significantly increased the levels of IL-8 mRNA transcript in comparison to untreated cells ($p < 0.0001$). The pretreatment of RPMI 2650 with U0126 (25 μM) significantly decreased the levels of IL-8 mRNA in the cells stimulated with rhIL-17A compared to the cells stimulated with rhIL-17A alone ($p < 0.0001$). Furthermore, the pretreatment of RPMI 2650 with HMW-HA ($p < 0.0001$) and MMW-HA ($p < 0.01$)significantly decreased the levels of IL-8 mRNA in the cells stimulated with rhIL-17A compared to the cells stimulated with rhIL-17A alone. Conversely, the pretreatment of the RPMI 2650 with LMW-HA did not control the activity of rhIL-17A on the levels of IL-8 mRNA (Figure 6).

3.7. Effect of HAdase HMW-HA on ROS Production, NOX-4, and IL-8 Synthesis. ROS production was significantly increased in RPMI 2650 treated with HMW-HA digested with HAdase (HAdase HMW-HA) in comparison to RPMI 2650 treated with HMW-HA before the stimulation with rhIL-17A for 6 hrs (Figure 7(a)). NOX-4 and IL-8 synthesis were significantly increased in RPMI 2650 treated with HMW-HA digested with HAdase (HAdase HMW-HA) in comparison to RPMI 2650 treated with HMW-HA before the stimulation with rhIL-17A for 18 hrs (Figures 7(b) and 7(c)). However, we underlined that RPMI 2650 treated with rhIL-17A and HAdase HMW-HA showed statistically significant lower levels of ROS, NOX-4, and IL-8 than the cells treated with rhIL-17A alone (Figures 7(a), 7(b), and 7(c)).

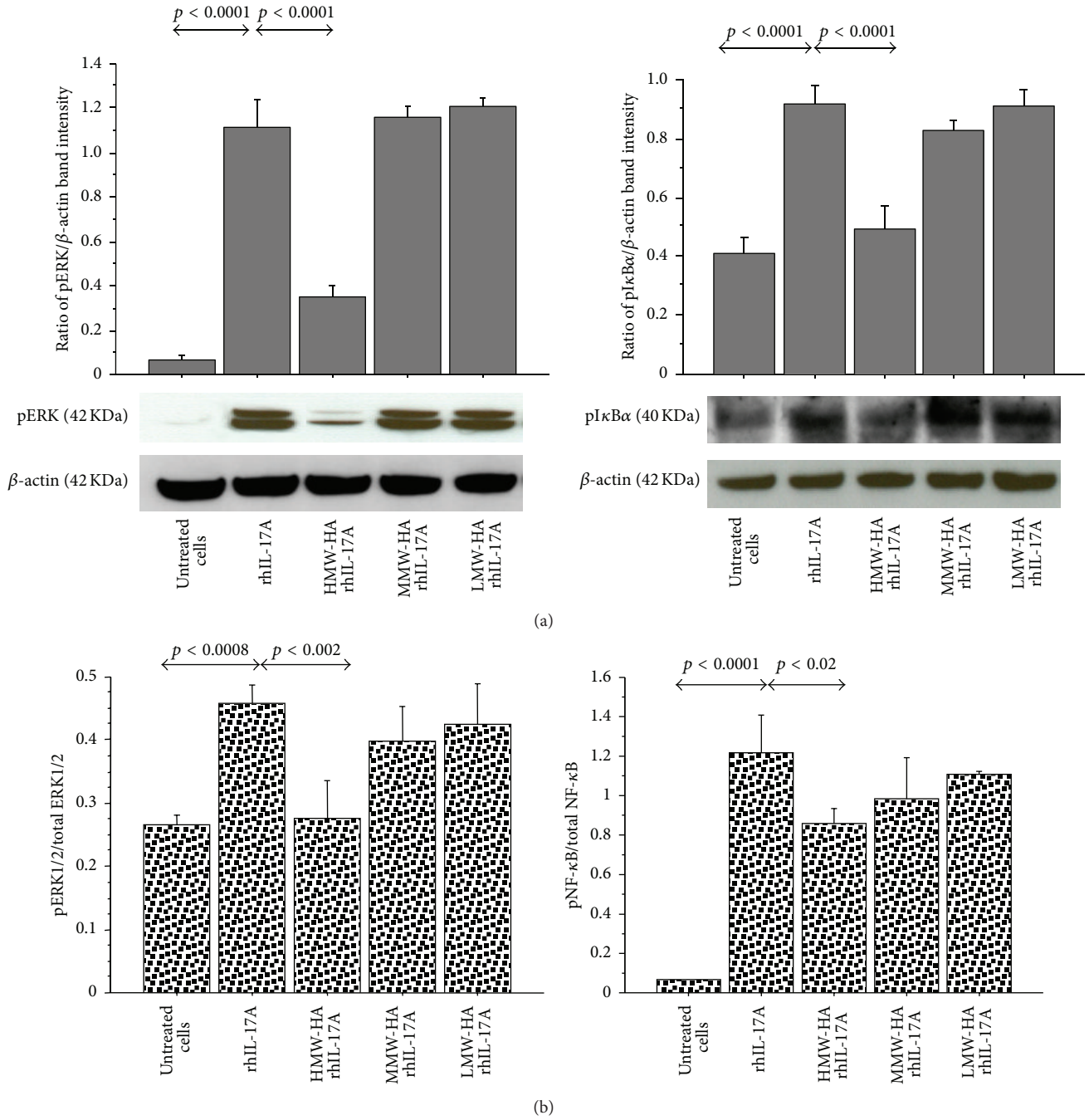

Figure 3: Effect of HMW-HA, MMW-HA, and LMW-HA on ERK1/2 and NF-κB signal pathway in RPMI 2650 cells stimulated with rhIL-17A. The cells were preincubated with HMW-HA (100 μg/mL), MMW-HA (100 μg/mL), and LMW-HA (100 μg/mL) for 1 h and then stimulated with rhIL-17A (20 ng/mL) for 30 min; (a) pERK and pIκBα protein expression were evaluated in the cell lysates by western blot. The bars represent the ratio of band intensity and β-actin of 3 separate experiments. Representative gel images of pERK, pIκBα, and β-actin are shown; (b) the activation of ERK1/2 and NF-κB for each experimental condition was tested for the pERK1/2/total ERK1/2 ratio and pNF-κB/total NF-κB ratio by ELISA and normalized for protein content. ANOVA with Fisher's test correction was used for the analysis of the data. $p < 0.05$ was statistically significant.

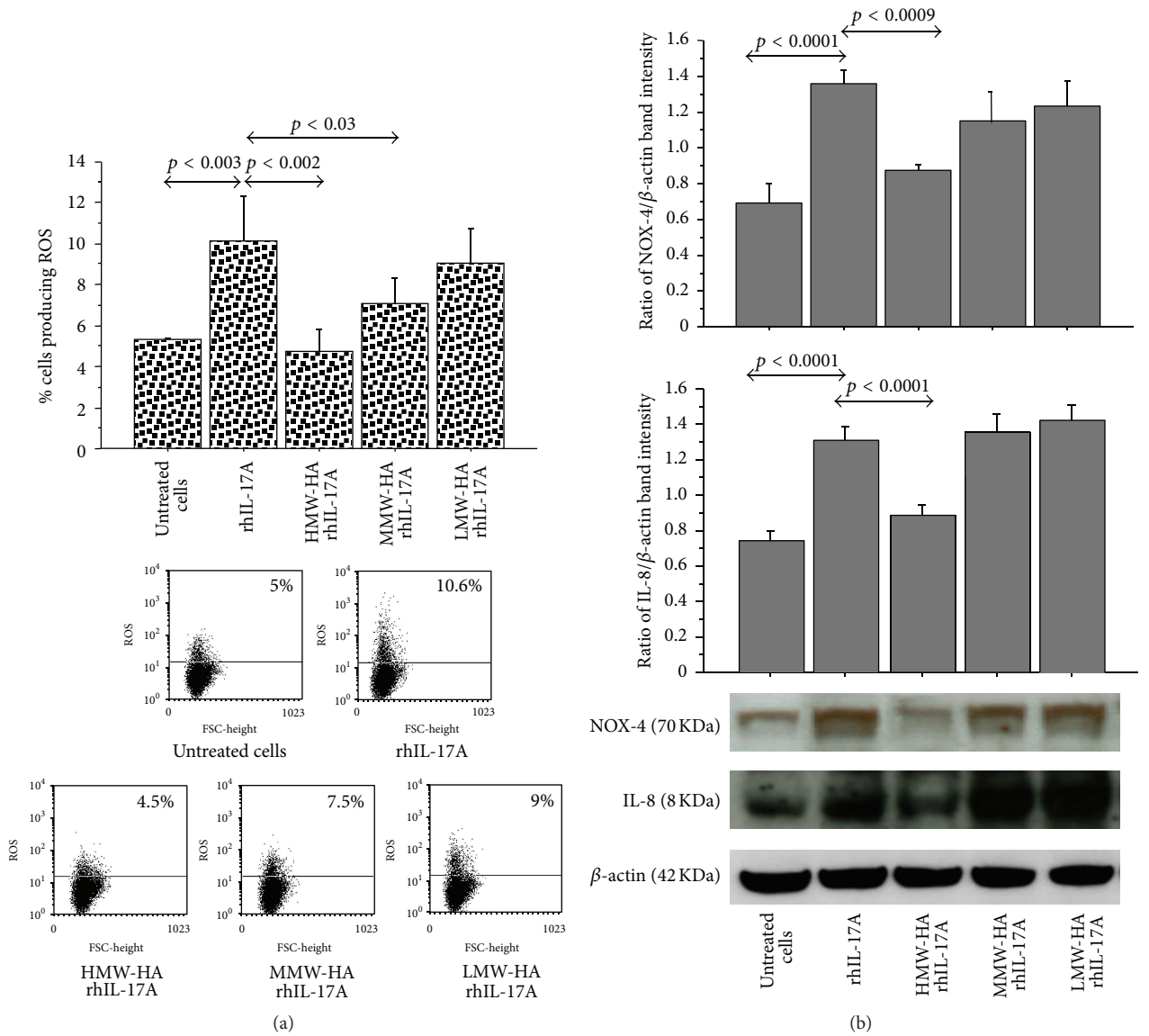

Figure 4: Effect of HMW-HA, MMW-HA, and LMW-HA in RPMI 2650 cells stimulated with rhIL-17A. (a) The cells were preincubated with HMW-HA (100 μg/mL), MMW-HA (100 μg/mL), and LMW-HA (100 μg/mL) for 1 h and then stimulated with rhIL-17A (20 ng/mL) for 6 hrs. ROS production was evaluated in the cells by flow cytometry. The bars expressed mean ± SD of 3 separate experiments. Representative flow cytometry is shown; (b) the cells were preincubated with HMW-HA (100 μg/mL), MMW-HA (100 μg/mL), and LMW-HA (100 μg/mL) for 1 h and then stimulated with rhIL-17A (20 ng/mL) for 18 hrs. NOX-4 and IL-8 protein synthesis were evaluated in the cell lysates by western blot. The bars represent the ratio of band intensity and β-actin of 3 separate experiments. Representative western blot is shown. ANOVA with Fisher's test correction was used for the analysis of the data. $p < 0.05$ was statistically significant.

4. Discussion

This study suggests the potential role of HMW-HA rather than MMW-HA as coadjuvant of the classic anti-inflammatory treatment during the nasal inflammatory and oxidative process IL-17A mediated. Indeed, our current findings identified the potential ancillary role of HMW-HA in the regulation of oxidative stress (ROS, NOX-4) and IL-8 synthesis generated by epithelial cells during nasal inflammation. Particularly in our *in vitro* model, we identify that HMW-HA might be able to control the mechanism of oxidative stress and inflammation blocking the ERK1/2 intracellular signal pathway activation involved in the NF-κB transcriptional mechanism regulation (Figure 8: graphical abstract).

IL-17 cytokines promote tissue inflammation via the induction of other proinflammatory cytokines and chemokines. Moreover, several studies in humans have demonstrated that Th17 immunity is involved in the pathogenesis of allergic diseases [22] with a potential role in the severity of the disease [23, 24]. The activity of IL-17A is mediated by IL-17 receptor (IL-17R) expressed by both blood cells and structural cells including T-cells and the airway epithelial cells [25].

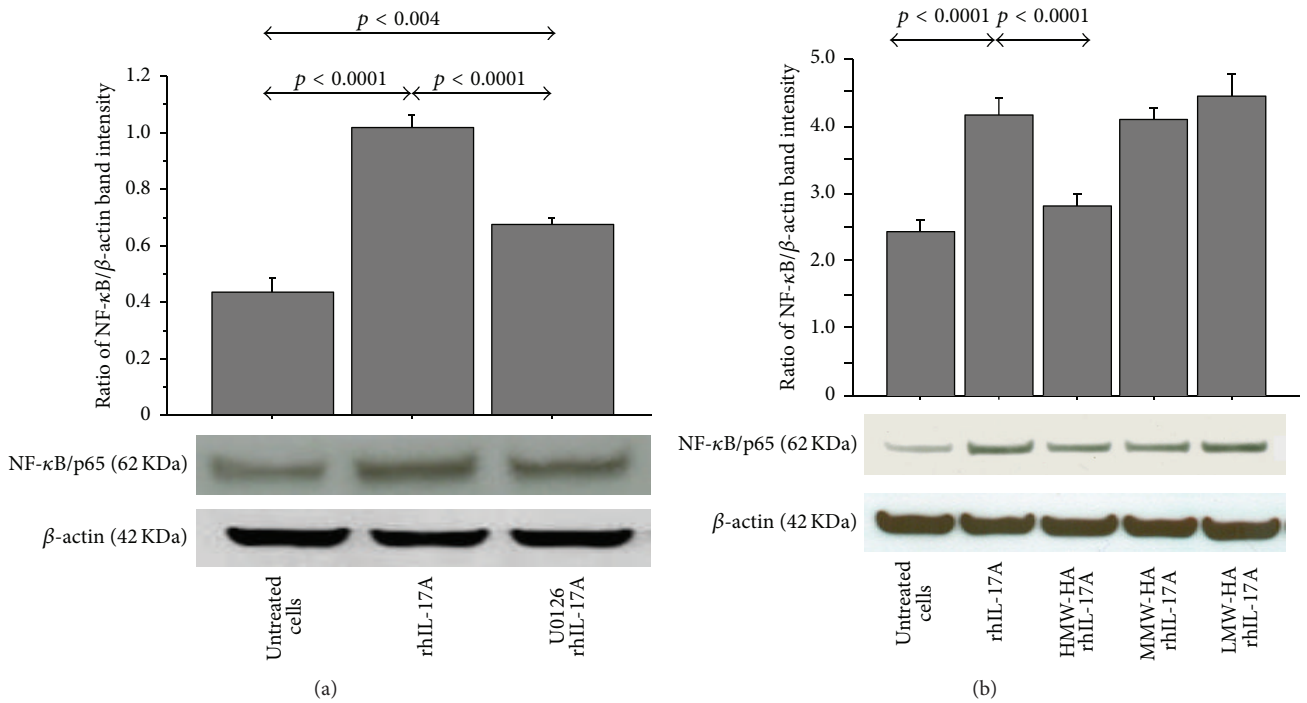

Figure 5: Effect of U0126, HMW-HA, MMW-HA, and LMW-HA on nuclear translocation of NF-κB in RPMI 2650 cells stimulated with rhIL-17A. The cells were preincubated (a) with U0126 (25 μM) or (b) with HMW-HA (100 μg/mL), MMW-HA (100 μg/mL), and LMW-HA (100 μg/mL) for 1 h and then stimulated with rhIL-17A (20 ng/mL) for 30 min; NF-κB was evaluated in nuclear cell lysate by western blot. The bars represent ratio of band intensity and β-actin of 3 separate experiments. Representative gel images of NF-κB and β-actin are shown. ANOVA with Fisher's test correction was used for the analysis of the data. $p < 0.05$ was statistically significant.

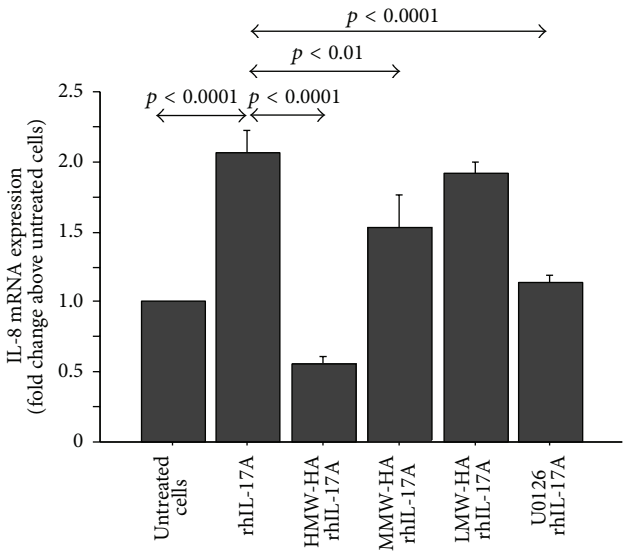

Figure 6: Effect of HMW-HA, MMW-HA, LMW-HA, and U0126 on IL-8 mRNA transcript. The cells were preincubated with HMW-HA (100 μg/mL), MMW-HA (100 μg/mL), LMW-HA (100 μg/mL), and U0126 (25 μM) for 1 h and then stimulated with rhIL-17A (20 ng/mL) for 18 hrs to study IL-8 mRNA. mRNA levels were quantified by quantitative Real-Time PCR (see Materials and Methods for details). The bars represent the mean ± SD of 3 separate experiments. ANOVA with Fisher's test correction was used for the analysis of the data. $p < 0.05$ was statistically significant.

In this scenario, IL-17A in allergic rhinitis and asthma may cover both the innate and the adaptive aspects representing the crucial crosstalk between immune system and structural cells such as fibroblasts and airway epithelial cells [26, 27]. On the other hand, higher levels of IL-17A were observed in the nasal wash from children with allergic rhinitis compared to healthy control [2], suggesting the relevant action of IL-17A in the nasal inflammation.

RPMI 2650 cell line used in this study has been shown to closely resemble normal human upper airway epithelium with respect to its karyotype, cytokeratin expression, and the presence of mucoid material on the cell surface and was previously used to study interactions of the airway epithelium with cytokines and allergens [21, 28, 29]. The use of RPMI 2650 nasal epithelial cells in the current study reflects our intent to understand the molecular and signaling underpinnings of the activity of IL-17A on nasal epithelial cells and whether the HMW-HA can protect the nasal epithelium from IL-17A mediated inflammation.

Nuclear factor- (NF-) κB, which consists of p50 and p65 subunits, is pivotal in the regulation of many genes including cytokines, chemokines, and adhesion molecules. Activation of NF-κB is dependent on the phosphorylation and degradation of IκB, an endogenous inhibitor that binds to NF-κB in the cytoplasm. The released NF-κB then translocates to the nucleus where it binds to specific NF-κB DNA binding sites and initiates gene expression. NF-κB activates

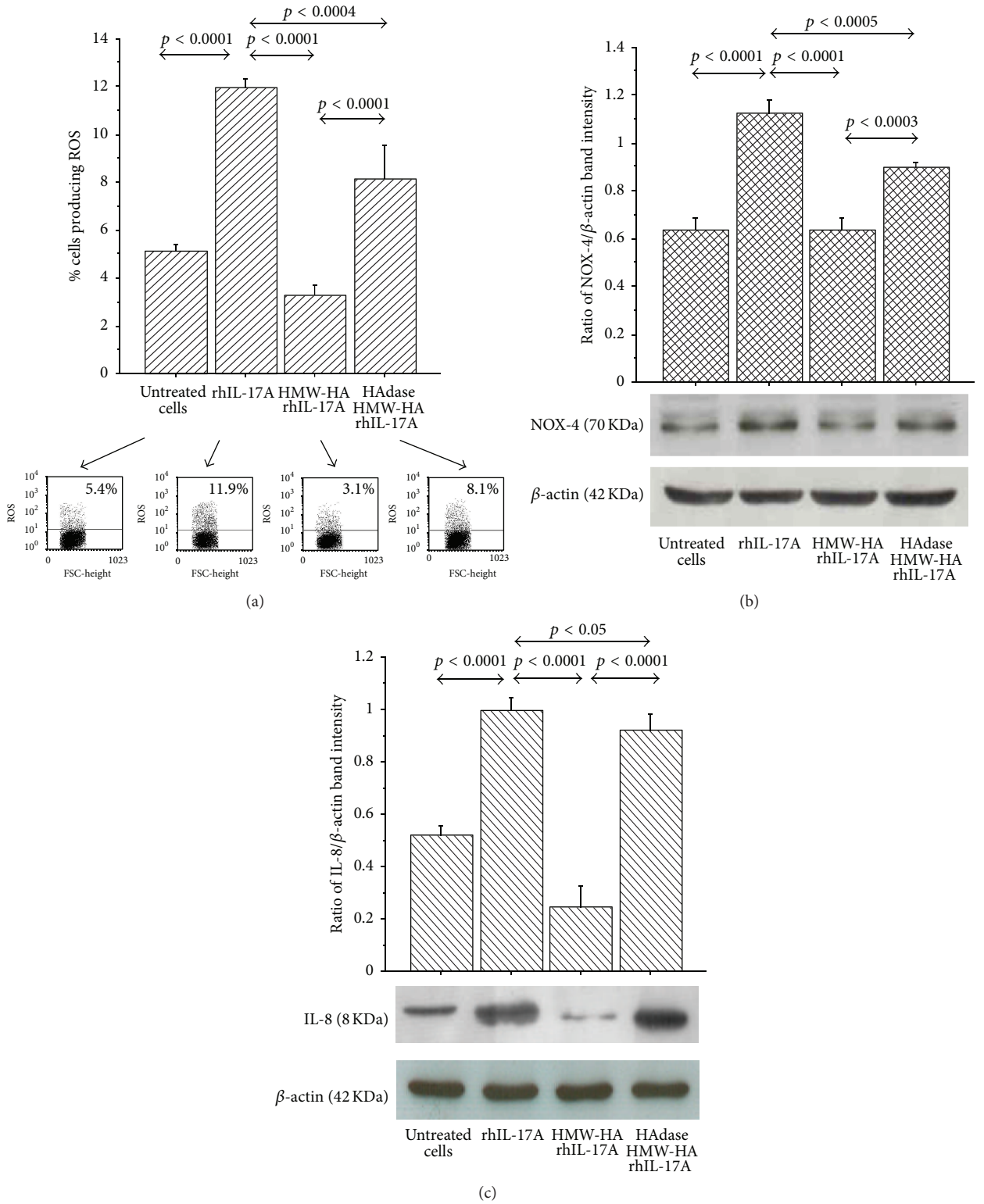

Figure 7: Specific effect of HMW-HA in RPMI 2650 cells stimulated with rhIL-17A. HMW-HA was incubated with HAdase 2.5 units/100 μg (60°C for 72 hrs plus 65°C for 10 min). The cells were preincubated with HMW-HA (100 μg/mL) or HAdase treated HMW-HA for 1 h and stimulated with rhIL-17A (20 ng/mL) for 6 hrs for ROS or 18 hrs for NOX-4 and IL-8. (a) ROS production was evaluated in the cells by flow cytometry. Representative flow cytometry is shown. The results were expressed as the mean ± SD of 3 separate experiments; (b-c) NOX-4 and IL-8 protein synthesis were evaluated in the cell lysates by western blot. The bars represent ratio of band intensity and β-actin of 3 separate experiments. Representative western blot is shown. ANOVA with Fisher's test correction was used for the analysis of the data. $p < 0.05$ was statistically significant.

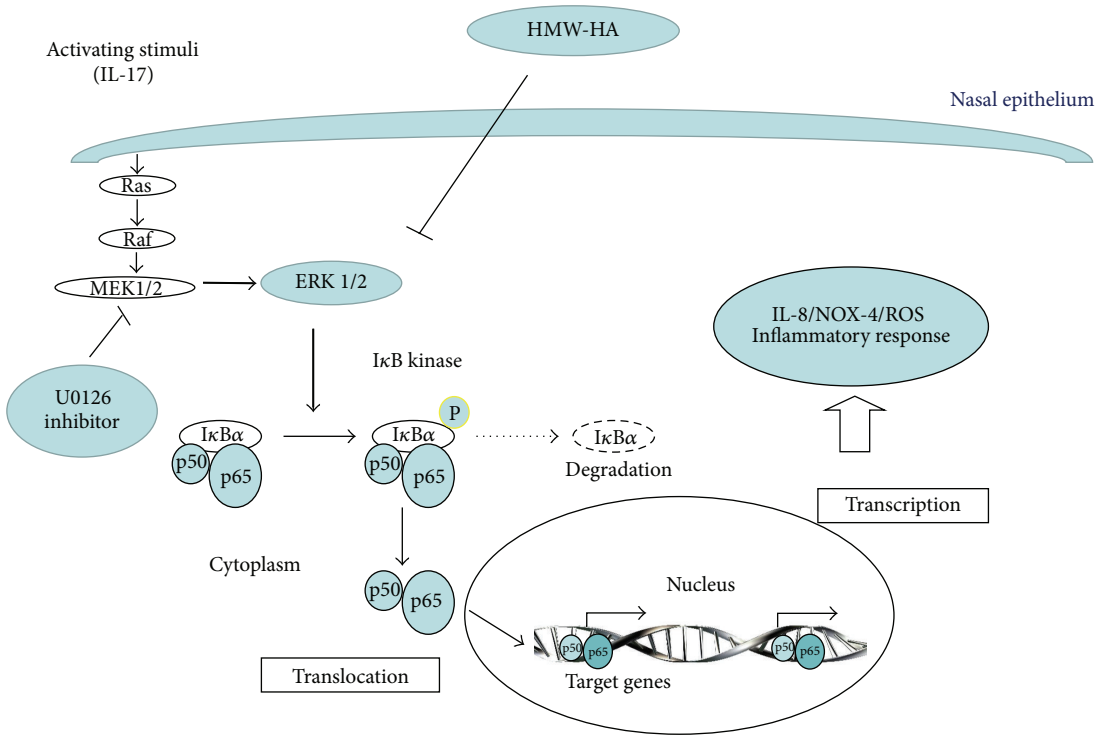

Figure 8: Graphical abstract of the study.

gene expression from NF-κB sites in association with the transactivation domains located in the carboxyl-terminus of the p65 protein [30, 31]. ERK1/2 and NF-κB play an important role in IL-17A-induced cytokine in human airway smooth muscle cells *in vitro* [32]. We demonstrated that IL-17A is able to activate ERK1/2 and IκBα phosphorylation, together with the nuclear translocation of NF-κB reduced using a specific inhibitor of MEK1 and MEK2 MAP kinase kinase inhibitor U0126 in nasal epithelial cells. These findings might suggest that IL-17A activity generates the NF-κB translocation to the nucleus via MEKK1 signaling cascades, promoting an increase of NF-κB at binding sites of genes involved in nasal epithelial cells activation during airway inflammation.

NOX-4, localized mainly in the epithelial layer, may play an important role in reactive oxygen species production, contributing to the oxidative stress in allergic rhinitis and nasal polyp tissues [33]. NOX family is a key component of the so-called redox signaling system regulating many cellular responses by intracellular ROS content [34]. IL-17A increases oxidative/nitrosative markers, likely via ERK1/2 downstream signaling in bronchial epithelial cells [35]. Accordingly, we found that IL-17A is able to activate ROS production and NOX-4 expression in nasal epithelial cells reduced by the use of the specific inhibitor U0126. These findings might suggest that IL-17A generates endogenous ROS via MEKK1 signaling cascades able to promote NF-κB activity at the binding site of NOX-4 in nasal epithelial cells.

Several *in vitro* studies have shown that recombinant IL-17A is able to induce IL-6, IL-8, granulocyte colony-stimulating factor (GCS-F), nitric oxide (NO), and prostaglandin E2 (PGE2) in airway epithelial cells [36]. RhIL-17A promotes the release of IL-8 in both nasal and bronchial epithelial cells [2] and IL-17A-induced IL-8 production p38, extracellular-signal-related kinase (ERK), and phosphoinositide-3-kinase (PI3K) pathways, and the latter appeared to be involved in IL-17A-induced GC insensitivity [37]. In our study, IL-17A is able to activate IL-8 production in terms of protein and mRNA in nasal epithelial cells that was reduced by the use of the specific inhibitor U0126. In this scenario, our findings might suggest that IL-17A generates IL-8 synthesis via MEKK1 signaling cascades able to promote NF-κB activity at the binding site of NOX-4 in nasal epithelial cells.

Hyaluronan is the main glycosaminoglycan (80%) produced by the respiratory mucosa and represents the main component of the film covering the upper airways. Together with chondroitin sulfate and heparan sulfate, it stratifies on the mucosal epithelial cells. Its topical use increases and improves the healing process, supporting the nasal functions of restoring and the mucosal tropism. Furthermore, the administration of hyaluronan can protect the sinonasal epithelium from inflammatory and surgical damage although there were no clear data about the cellular and molecular mechanism by which it can act [38]. Recent studies revealed increased amounts of total HA and LMW-HA in animal models of acute lung injury [39–42]. Furthermore, LMW-HA can induce the expression of several proinflammatory cytokines, including IL-8 [42–44]. Moreover, HMW-HA can inhibit LPS-activated PI3K/Akt pathway leading to downregulation of NF-κB with diminished IL-6 production through

interaction with ICAM-1 in lipopolysaccharide (LPS-) stimulated U937 macrophages [43]. Additionally, HA suppresses advanced glycation end product (AGE-) induced expression of proinflammatory cytokines and NF-κB nuclear translocation in J774 mouse macrophages [44]. ERK1/2 and NF-κB inhibitors significantly abrogate the response to HMW-HA, suggesting an important role for HA in the regulation of epicardial cell fate via activation of MEKK1 signaling cascades [34]. Accordingly with these observations, we demonstrated that HMW-HA, rather than MMW-HA or LMW-HA, is able to downregulate the activation of ERK1/2 and IκBα phosphorylation and nuclear translocation of NF-κB as well as the related ROS, IL-8, and NOX-4 protein. These findings suggest that HMW-HA with the involvement of MEKK1 signaling cascades and NF-κB activity in nasal epithelial cells might be able to exercise a protective role in nasal inflammation and did not increase inflammation. In contrast, in our *in vitro* model, LMW-HA or MMW-HA did not affect the biological activity of IL-17A in nasal epithelial cells. Finally, the use of hyaluronidase in the experimental condition, responsible for the HMW-HA fragmentation in nasal epithelial cells culture, reduced the protective action of HMW-HA confirming its specific activity. Additionally, we observed that RPMI 2650 treated with rhIL-17A and HMW-HA digested with HAdase showed statistically significant lower levels of ROS, NOX-4, and IL-8 than the cells treated with rhIL-17A alone. This data suggest a residual activity of HMW-HA in the presence of *Streptomyces* HAdase. This action is in accord with a random endolytic action pattern of *Streptomyces* HAdase due to the presence of some resistant (less susceptible to enzymolysis) sites, ascribed to restricted enzyme access, in the HA polymer [45]. Nevertheless, our findings clearly provide data on a protective effect of HMW-HA. In this scenario, our results clearly support the protective peculiarity of HMW-HA in the control of the nasal epithelial cell activation during the inflammatory process generated by IL-17A. However, further studies might be necessary to better clarify whether the protective activity of HMW-HA is associated with chemical-physical mechanism or with receptor activity in the nasal epithelium. Finally, while IL-8 mRNA transcript reflects the related protein expression, NOX-4 mRNA transcript was not modulated in our study. Accordingly, it was observed that NOX-4 mRNA is regulated at both transcriptional and posttranscriptional levels, and often, the level of NOX-4 mRNA does not accurately reflect NOX-4 protein expression and functions [46].

5. Conclusions

This study identified for the first time that HMW-HA is able to downregulate the mechanism of uncontrolled oxidative stress and inflammation typical of upper airway diseases. Indeed, our results provided encouraging evidence to support possible beneficial effects of HMW-HA in the care of patients with altered function of nasal epithelial cells, supporting its potential ancillary role as coadjuvant of the classic anti-inflammatory treatment of the nose. However, additional clinical studies should be performed to assess the usefulness of these observations in clinical practice.

List of Abbreviations

HMW:	High molecular weight
MMW:	Medium molecular weight
LMW:	Low molecular weight
HA:	Hyaluronan
IL-17A:	Interleukin-17A
IL-8:	Interleukin-8
ROS:	Reactive oxygen species
NOX-4:	NADPH oxidase
MAPK:	Mitogen-activated protein kinase
MAPKK:	MAP kinase kinase
NF-κB:	nuclear factor kappa-light-chain-enhancer of activated B-cells
ERK1/2:	extracellular-signal-regulated kinases
IκBα:	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
U0126:	1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto) butadiene monoethanolate
PMA:	Phorbol 12-myristate 13-acetate
DCFH-DA:	Dye 2,7-dichlorofluorescein diacetate
HAdase:	Hyaluronidase.

Competing Interests

The authors of the paper, Luca Cavalieri and Eleonora Ingrassia, are employees of Chiesi Farmaceutici S.p.A., Parma, Italy. There are no other competing interests for this study.

Authors' Contributions

Giusy Daniela Albano, Anna Bonanno, and Mirella Profita conceived the study and designed the experiments. Caterina Di Sano, Rosalia Gagliardo, Liboria Siena, Loredana Riccobono, and Giusy Daniela Albano performed the technical procedures. Mirella Profita provided the interpretation of data and wrote the paper. Luca Cavalieri and Eleonora Ingrassia revised the final draft of the paper. All authors read and approved the final version of the paper.

Acknowledgments

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