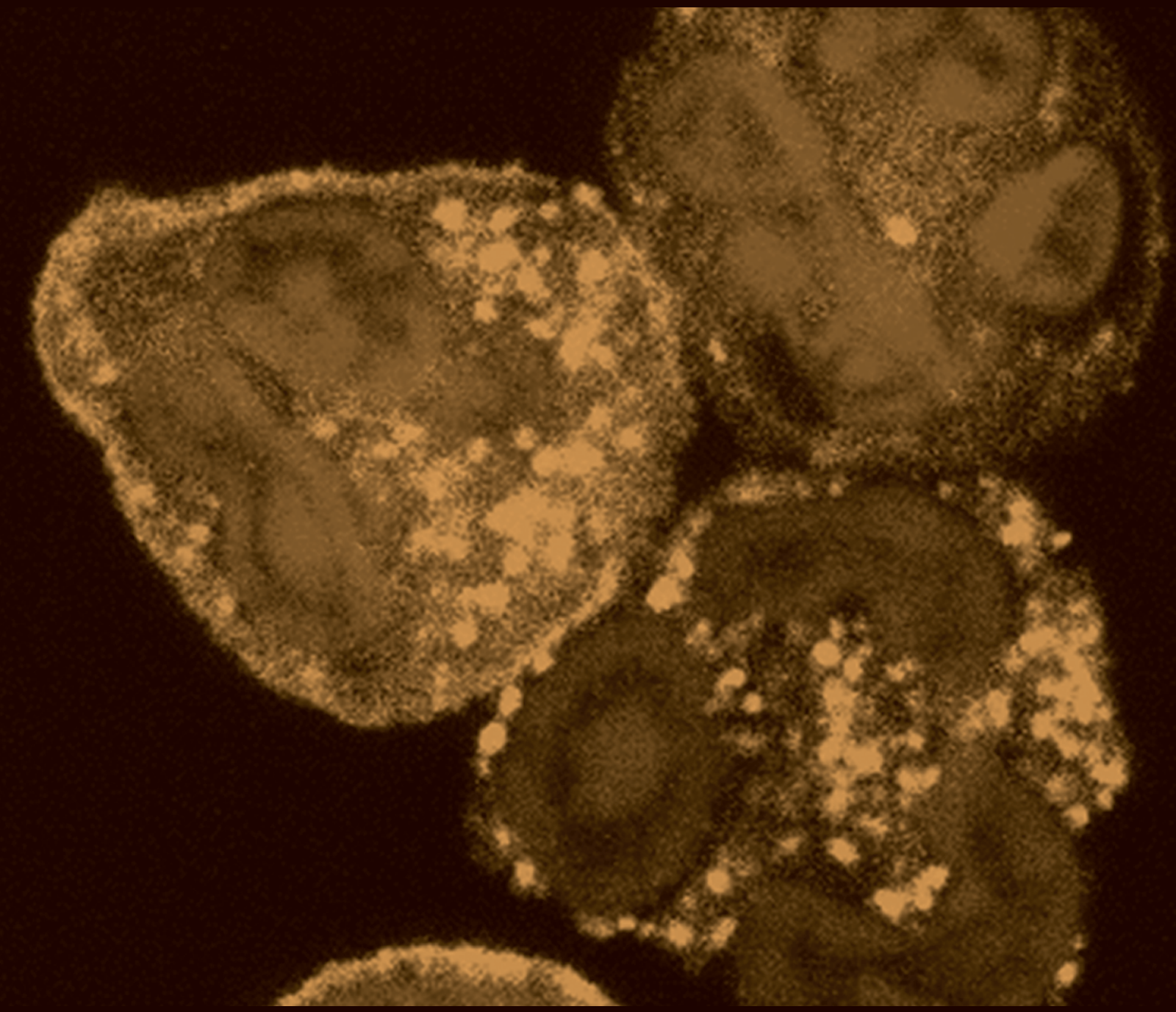
Mediators of Inflammation

Lipid Mediators and the Immune Response

Guest Editors: Lúcia Helena Faccioli, David M. Aronoff, Ruxana Sadikot, Carlos Henrique Serezani, and Nicolas Flamand



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Review Article

Prostaglandin E_2 and the Suppression of Phagocyte Innate Immune Responses in Different Organs

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The local and systemic production of prostaglandin E_2 (PGE_2) and its actions in phagocytes lead to immunosuppressive conditions. PGE_2 is produced at high levels during inflammation, and its suppressive effects are caused by the ligation of the E prostanoid receptors EP_2 and EP_4, which results in the production of cyclic AMP. However, PGE_2 also exhibits immunostimulatory properties due to binding to EP_3, which results in decreased cAMP levels. The various guanine nucleotide-binding proteins (G proteins) that are coupled to the different EP receptors account for the pleiotropic roles of PGE_2 in different disease states. Here, we discuss the production of PGE_2 and the actions of this prostanoid in phagocytes from different tissues, the relative contribution of PGE_2 to the modulation of innate immune responses, and the novel therapeutic opportunities that can be used to control inflammatory responses.

1. General Considerations

Prostaglandins (PGs) are lipid mediators derived from arachidonic acid (AA) metabolism via the activation of the cyclooxygenase (COX) pathway, that regulates inflammation, immune response, hematopoiesis, tissue injury and repair, and bone resorption. PGs are found in most tissues and organs, and the variety of effects that they can elicit reflects the presence of specific PG receptors in many cell types. Upon cell activation by microbial products, cytokines, and opsonins, cytosolic phospholipase A_2 (PLA_2) is activated and recruited to hydrolase plasma cell phospholipids. Once it is released from the membrane, AA is rapidly converted into PGs by cells expressing prostaglandin H synthase (COX). At least two COX isoforms exist, the constitutive (COX-1) and inducible (COX-2) isoforms. COX-1 is expressed in many cell types distributed throughout the body, whereas COX-2 expression is highly restricted under basal conditions and upregulated during inflammation in different cell types [1] (see Figure 1). COX proteins are the major targets of nonsteroidal anti-inflammatory drugs (NSAIDs).

COX-2 is transcriptionally regulated by mediators that act through phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase1/2 (ERK1/2), and p38, and the activation of COX-2 culminates in the activation of the transcription factors, nuclear factor kappa B (NFκB), activator protein (AP-1) and the cAMP response element-binding (CREB) [2, 3]. Therefore, COX-2 activity is induced by a variety of proinflammatory cytokines and growth factors and by one of its products, PGE_2. Conversely, COX-2 expression is inhibited by glucocorticoids and interleukin (IL)-4. Both COX-1 and COX-2 are present in the active state in the endoplasmic reticulum and the nuclear envelope. These enzymes convert AA to the unstable endoperoxide

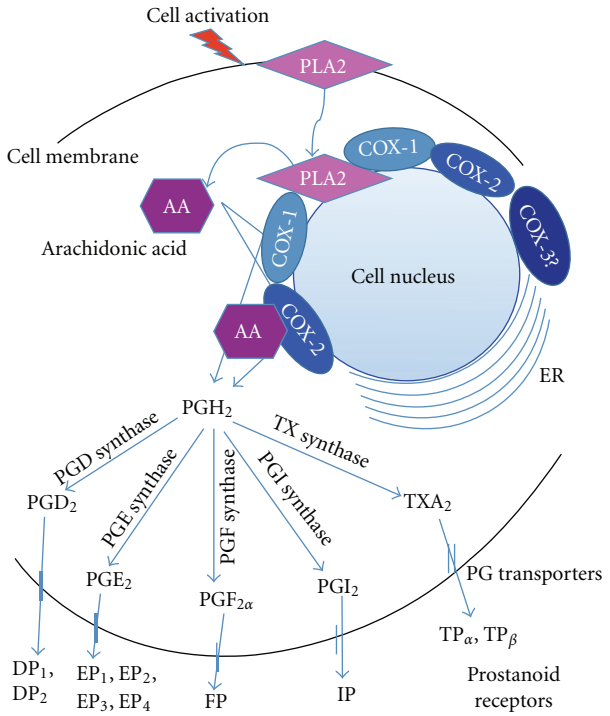

FIGURE 1: Prostanoid biosynthesis and receptors. Upon cell stimulation, PLA2 is activated, and (AA) is released from the cellular membranes. AA is then metabolized by COX-1 or COX-2 in different cellular compartments and further metabolized by different synthases, which leads to the generation of different prostanoids. Once the product is formed, different prostanoids are transported outside the cells to bind to their respective receptors. (PG prostaglandin; Tx thromboxane; PGJ_2 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; Cox-1/2 cyclooxygenase-1/2; PGDS, PGES, PGFS, and PGIS prostaglandin $D_2/E_2/F_2/I_2$-synthase; PGIS prostacyclin synthase; TxAS thromboxane A_2 synthase; PGER prostaglandin E2 9-reductase).

PGH_2, which is converted by specific synthases to the five following biologically active prostanoids: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), and thromboxane A2 (TXA_2). There are several PGE synthases, and one of these synthases (mPGES-1) is a highly inducible microsomal enzyme that acts downstream of COX to catalyze the conversion of PGH_2 to PGE_2 [4–6] (Figure 1).

PGE_2 is a potent mediator of inflammation that induces both pro- and anti-inflammatory effects and signals via four different E prostanoid (EP) receptors, EP_1-EP_4. The EP receptors are member of a family of G protein-coupled receptors (GPCRs). EP_1 signals through $G\alpha_q$, which leads to increased levels of Ca^{2+}. EP_2 and EP_4 signal through $G\alpha_s$, which leads to increased cAMP levels. EP_3 primarily signals through $G\alpha_i$, which leads to decreased cAMP levels [7] (Figure 2).

The distribution and relative expression of these four receptor subtypes provide an elegant system that can account for the ability of PGE_2 to evoke pleiotropic and sometimes opposing bioactions that are tissue- and cell-type specific.

Although PGE_2 is commonly considered to be a potent proinflammatory mediator [8], its role as a mediator of anti-inflammatory responses is now being studied [9, 10]. The anti-inflammatory response opposes the host inflammatory response, which potentially limits collateral damage to neighboring cells and tissues and aids in the resolution of inflammation after the pathogens are contained [11]. This dual effect depends on the cell type, the tissue compartment, the state of cellular activation, and the particular expression of the signaling-EP receptors. The existence of four subtypes of receptors that signal differently and can be expressed in different combinations in a single cell explains the multiplicity of biological responses that are elicited by PGE_2 and how these responses may differ among cells and tissues. This paper reviews the recent knowledge regarding PGE_2 synthesis and its modulatory effect on innate immune responses in different tissues.

2. Lung

The synthesis of PGE_2 occurs in several different cellular types within the airways, such as epithelial cells, fibroblasts, vascular endothelial cells, and leukocytes [12]. The leukocytes that can synthesize PGE_2 include the alveolar macrophages (AMs), neutrophils, follicular dendritic cells, and T cells. The relative capacity of these cells to produce PGE_2 is shown in Table 1. The AMs represent a major source of PGE_2 during microbial infection [13], whereas alveolar epithelial cells and pulmonary fibroblasts also represent an important source of PGE_2 in the lungs [14]. High levels of PGE_2 are produced in AMs following the lipopolysaccharide (LPS)-and granulocyte/macrophage colony-stimulating factor (GM-CSF)-dependent expression of the inducible form of COX-2 [15]. Several mediators and signal transduction pathways are involved in the modulation of the synthesis and release of PGE_2 by these cells. The inhibition of endogenous rat AM-producing transforming growth factor (TGF)-β enhances PGE_2 synthesis, while the expression of LPS-induced COX-2 and PGE_2, which are released by human AMs, is upregulated following the inhibition of PI3K activity [3]. AMs also produce increased PGE_2 after bone marrow transplantation [16]. Although neutrophils are considered to be the main producers of leukotriene B_4 (LTB_4) (5-lipoxygenase-derived lipid mediator), few studies have attempted to evaluate the ability of lung neutrophils to produce prostanoids. In fact, the majority of studies is focused on the peritoneal and peripheral blood-derived neutrophils [17]. One of these studies demonstrated that lung PMNs (but not AMs) from mice that received bone marrow transplants synthesized pronounced levels of PGE_2 when compared with cells from control mice [16]. In general, the *in vitro* synthesis of the cytokine-induced PGE_2 by neutrophils involves the activation and novel synthesis of COX [18]. In addition, while PGE_2 synthesis is well documented in human monocyte-derived immature dendritic cells (DCs) [19], no studies to date have demonstrated the particular capacity of lung DCs to produce this mediator.

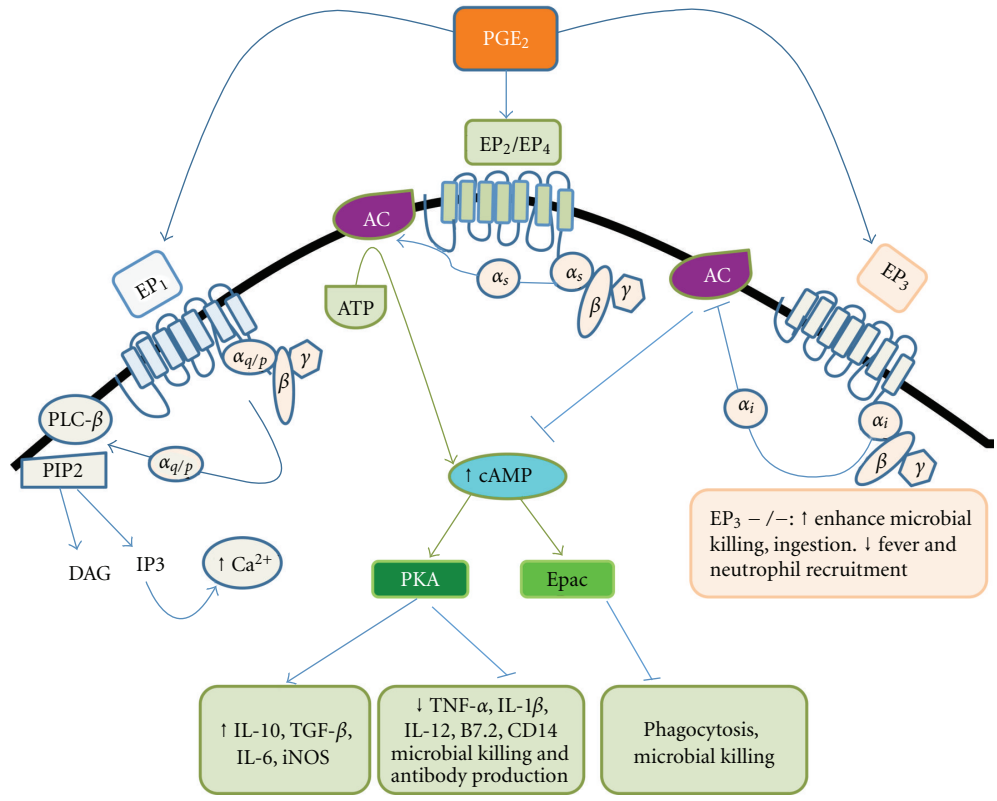

FIGURE 2: PGE_2 receptors and their actions in macrophages. PGE_2 produced during inflammatory conditions binds to EP_2, EP_4, EP_3, or EP_1. EP_2 and EP_4 are coupled to $G\alpha_s$, and the binding of PGE_2 to these G protein-coupled receptors (GPCRs) induces a conformational change that results in the liberation of the $G\alpha_s$ subunit from the $G\beta\gamma$ subunit complex. The binding of the $G\alpha$ subunit to adenylyl cyclase (AC) either stimulates ($G\alpha_s$) or inhibits ($G\alpha_i$, via EP_3 signaling) the enzyme's generation of cAMP. The production of cAMP is also regulated by microbial pathogens. Downstream cAMP signaling is mediated by its interactions with effector molecules, such as protein kinase A (PKA), or exchange proteins that are directly activated by cAMP (Epac), which have been shown to modulate phagocyte functions. Depicted here is a pattern for alveolar macrophages in which specific antimicrobial functions are differentially regulated by specific cAMP effectors.

PGE_2 produced in the lungs elicits a wide variety of effects [1]. The effects vary from the induction of tissue repair and pulmonary vascular remodeling [20] to the regulation of immune inflammatory responses [21].

AMs are the primary lung cells that are involved in the protection of the alveolar-blood interface and serve as the front line of cellular defense against respiratory pathogens [22] in both murine and human cells. AMs express all four types of EP receptors [23] and contribute greatly to the amount of PGE_2 produced in infected lungs [13] (Table 1). Monick and collaborators have demonstrated that LPS induces COX-2 expression and PGE_2 release in human AMs [3, 24].

The immunomodulatory effects of PGE_2 are largely caused by its ability to increase intracellular cAMP through the stimulatory $G\alpha_s$-coupled EP receptors EP_2 and EP_4 [25]. Increases in intracellular cAMP levels are transduced into cellular responses mediated by its effectors, cAMP-dependent protein kinase A (PKA), and the exchange protein directly activated by cAMP-1 (Epac-1) [26]. In phagocytes, the effects of PGE_2 are usually anti-inflammatory since PGE_2 has been demonstrated to inhibit the production of proinflammatory molecules and increase the secretion of anti-inflammatory cytokines, such as IL-10 [27]. In human AMs, PGE_2 potently inhibited LPS-induced tumor necrosis factor (TNF)-α through the activation of the EP_2 and EP_4 receptors [28]. The downmodulation of LPS-induced TNF-α by PGE_2 in rat AMs is dependent on cAMP signaling-dependent PKA activation since the selective PKA activating cAMP analog 6-Bnz-cAMP, but not the Epac-1 activating analog 8-pCPT-2-O-Me-cAMP, inhibits its production [29]. EP_2 signaling is also involved in the enhancement of LPS-induced nitric oxide (NO) by the activation of PKA rather than Epac-1 [30]. Exogenous PGE_2 can potentiate the synthesis of LPS-mediated IL-6 and IL-10 in rat AMs via AKAP10-(A-kinase anchoring protein-10-) mediated PKA signaling, while the suppression of TNF-α occurs via AKAP-8-anchored PKA-RII (PKA regulatory subunit type II) [30].

PGE_2 has also been shown to inhibit AM FcR-mediated phagocytosis by activating the EP_2 receptor, judged by the mimicked effect of the selective EP_2 agonist butaprost [23] or a specific Epac-1 agonist (8-pCPT-2′-O-Me-cAMP) [32]. Moreover, PGE_2 inhibits rat AM microbicidal activity and this effect was restored after treatment with indomethacin, EP_2, and EP_4 antagonists [31]. The role of EP_3 receptor activation-driven AMs was also studied in the context of

Table 1: Prostaglandin E_2 Synthesis and Receptor Expression in Leukocytes from different organs.

Type of compartment	Type of cells	Relative synthetic capacity	Receptor expression			
			EP_1	EP_2	EP_3	EP_4
Lung	Neutrophils	−	+	+[&]	+	+[&]
	Alveolar macrophages	+ + +	−	+ + +	+	++
	Dendritic cells	+*	+	++[&]	+	++[&]
Spleen	Neutrophils	−	ND	ND	ND	ND
	Macrophages	+*	ND	ND	ND	ND
	Dendritic cells	+	ND	ND	ND	ND
Bone	BMDM-derived	+ + +	+	+ + +	+	+ + +
	osteoclasts	+	+	++	+	++

Relative synthetic capacity is expressed by the number of plus (+) signs; a minus sign (−) characterizes no or a negligible synthetic capacity. Receptor expression is classified as positive (+), negative (−), minimal (±), or not determined (ND). *Synthesis of PGE_2 is relatively low in unstimulated conditions but is upregulated upon stimulation. [&]Receptor expression is upregulated during inflammatory stimulus.

pulmonary infection. Although the $G\alpha_i$-coupled EP_3 was thought to oppose the $G\alpha_s$-coupled EP_2 and EP_4 receptors, $EP_3^{-/-}$ mice were protected from bacterial induced death, which corroborates the increased ability of AMs to phagocytose and kills *Streptococcus pneumoniae* [33]. Through EP_2, PGE_2 was also involved in the mediation of the immunosuppressive response characterized by increased IL-10 synthesis and the impairment of neutrophil recruitment to the lungs during the ingestion of apoptotic cells (efferocytosis) by phagocytes [10]. As a suppressive mediator, PGE_2 inhibits AA release and LTB_4 synthesis in rat AMs by a mechanism independent of PLA_2 [34].

Human and mouse lung DCs are localized in the airway epithelium, lung parenchyma, visceral pleura, and bronchoalveolar lavage fluid (BALF) [35]. DCs exposed to PGE_2 exhibit a decreased capability to secrete proinflammatory cytokines [36]. They are in contact with many other cells in the lungs such as the airway epithelium, type II alveolar epithelial cells, AMs, pulmonary interstitial macrophages, (myo)fibroblasts, bronchus-associated lymphoid tissue (BALT) lymphocytes, nonadrenergic, noncholinergic (NANC) nerve endings, capillary endothelium, and mast cells. Although the particularly contribution of lung DC as producer of PGE_2 is still unknown, there are several studies using bone-marrow-derived DCs (BM-DCs) showing that their immunomodulatory function is highly regulated by mediators including PGE_2, potentially produced by neighboring cells in the lungs. BM-DCs exposed to PGE_2 present decreased ability to secrete proinflammatory cytokines [36]. The importance of lung DC modulation by PGE_2 is highlighted considering DC as the mediator cell of the adaptative immune response and the lungs as an important local tissue for airway microbial defenses [37].

Lung PMNs are the primary cells recruited to the lungs during acute lung injury [38]. LPS is an important inducer of the inflammatory response by its activation of Toll-like receptor 4 (TLR4). After binding to TLR4, LPS triggers the synthesis of chemoattractants that induce PMN migration at sites of inflammation, such as the lung [39]. The overproduced PGE_2 by lung PMNs from bone marrow transplantation mice is involved to the decreased ability of PMN to kill *Pseudomonas aeruginosa*, an effect restored by the PG inhibition with indomethacin [16]. However, evaluation of EP signaling in the PGE_2-mediated impaired host defense by lung PMMs is much less appreciated.

Due to the low yield of murine alveolar macrophages, one plausible alternative to study PGE_2 synthesis/actions is the use of alveolar macrophage cells lines. However, a very limited number of studies have been done to identify the profile of PGE_2 synthesis and actions in this cell line. Here, we are summarizing some of the key findings regarding the expression of COX mRNA and protein in MH-S murine alveolar macrophages. MH-S is a murine alveolar macrophage cell line transformed by SV40 obtained from Balb/c mice and displays several properties of primary AM, such phagocytic capacity and expression of Mac-1 antigen, major histocompatibility complex class II, the CR3 receptor, and the Fc receptor Mbawuike and Herscowitz, 1989 to [40]. LPS-stimulated MH-S cell line promotes robust increment of COX-2 and large amounts of PGE_2 (Joo et al., 2005 to [41]; Chen et al., 2007 to [42]). Luteolin, a flavonoid that exhibits anti-inflammatory properties, is shown to inhibit COX-2 gene expression and PGE_2, IL-6, TNF-α, and iNOS production in LPS-activated MH-S cells by decreasing NF-κB and AP-1 activation Chen et al., 2007 to [42]. In this context, LPS or overexpression of IKKβ is reported to activate NF-κB signaling and COX-2 expression, which was impaired after ectopic expression of hepatitis C virus in MH-S cells Joo et al., 2005 to [41]. However, so far there are no reports regarding EP receptors expression profile and the relative role of individual receptor in MH-S cells.

3. Spleen

Splenic macrophages, DCs, and lymphocytes contribute to PGE_2 synthesis in the spleen [43]. In splenic tissues, mPGES-1 accounts for the majority of basal (COX1-dependent) PGE_2 synthesis, and the *in vivo* mPGES-1 deletion abolished LPS-inducible PGE_2 synthesis [44]. Normal splenic macrophages produce low levels of PGE_2 when compared with bone-marrow-derived macrophages (BMDM; Table 1),

AMs, and peritoneal macrophages [45]. However, high levels of this mediator are produced by splenic macrophages in chronic inflammatory conditions, such as mycobacterial infection [46]. It has been shown that the formation of PGE_2-producing splenic macrophages is dependent on the radiosensitive bone marrow cells [47]; the precursors migrate from the bone marrow cells to the spleen to become mature cells [48]. Splenic DCs appear phenotypically immature and mature after microbial stimuli [37]. The phenotype seems to be determined by other suppressive mediators, including NO, TGF-β, 1α, 25 dihydroxyvitamin D3 (vitamin D) and PGE_2 produced by antigen-presenting cells (APCs) such as macrophages and DCs [49]. To date, no reports have described EP expression in splenic DCs; most studies are focused on bone-marrow-derived DCs (BM-DCs) [50]. These cells express all four EP receptors [51] that can induce different effects, including DC generation, migration, and maturation [52].

PGE_2-producing macrophages that are induced from mycobacterial stimuli interact closely with splenic lymphocytes to induce a shift from the Th1 to Th2 immune responses in a PGH_2 synthase-dependent manner [53]. This shift is based on the suppressive effect of the synthesis of Th1 cytokines, such as IL-1, IL-12, and interferon (IFN)-γ, but it does not affect Th2 cytokines [54]. The downmodulation of TNF-α synthesis by PGE_2 in *in vitro*-derived BM-DCs occurs through EP_2- and EP_4-induced signal transduction events [55]. It has also been shown that this signaling can upregulate IL-23 synthesis and downmodulate APC-produced IL-12 [56], which favors the expansion of IL-17-producing Th17 cells [57].

4. Bone

PGE_2 produced in the bone is primarily derived from osteoblasts, cells responsible for bone formation [58]. As shown in Table 1, mouse BMDMs, osteoclast precursors, and mature osteoclasts differentially express EP receptors. BMDMs express the EP_1, EP_2, EP3β, and EP_4 receptors, while mature osteoclasts only express the EP_1 receptor [59]. It was demonstrated that PGE_2 can stimulate cAMP levels in BMDMs but does not affect cAMP in mature osteoclasts; this result demonstrates that functional EP_2 and EP_4 receptors are inhibited in osteoclasts during its differentiation [59].

Osteoclasts are bone-resorbing multinucleated cells derived from the monocyte-macrophage lineage [60]. The differentiation and activation of osteoclasts are tightly regulated by osteoblasts through the release of receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [61], which are required for the differentiation of osteoclast progenitors into mature osteoclasts [62]. RANKL activation induces COX-2 expression in immature osteoclast by utilizing a Rac1-dependent NK-κB activation pathway; that results in PGE_2 synthesis and contributes to accelerated osteoclast differentiation [63].

In bone, PGE_2 is known to be an important local factor in the regulation of bone formation [64] and resorption [65]. PGE_2 acts in precursors and mature osteoclasts to regulate their function. PGE_2 can directly inhibit the bone-resorbing activity of osteoclasts. This inhibitory effect was dependent on an increase of intracellular cAMP caused by activator of adenylate cyclase (forskolin) and mimicked by the EP_2 and EP_4 agonists (butaprost and AE-604). In calvaria culture from EP_4 knockout mice, PGE_2 presented an impaired role in promoting bone resorption, whereas EP_2 agonist slightly restored bone resorption and EP_4 agonist did not [66].

5. Central Nervous System (CNS)

Although the immunoprivileged status of the CNS is well known, similar to any other organ, it is connected and engaged with the immune system to maintain tissue homeostasis. An excessive inflammatory status can promote several types of brain damage, which include ischemia and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [67].

The CNS typically contains low prostanoid levels. Specifically, PGE_2, PGD_2, and PGF_{2a} are associated with inflammatory responses [68]. Oddly, the COX-1 and COX-2 enzymes are both constitutively expressed in the CNS (in neurons, astrocytes, microglia and endothelia) [69], and a putative COX-3 enzyme, which is a splice variant of COX-1 that is denoted as COX-1b, is described in rodent and human neural tissues [70–72]. The PGE_2 levels in the CNS are enhanced during various neurological diseases, such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease [68].

Importantly, the proinflammatory stimuli that lead to brain injury further enhance COX-2 expression and therefore enhance PGE_2 synthesis. All three PGES isoforms are found in the CNS tissues, and the expression levels vary according to the cell type [73]. An elegant study demonstrated that brain PGE_2 synthesis is orchestrated by COX-1/COX-2/membrane-associated cPGES (cPGES-m) and by nuclear/perinuclear COX-2/mPGES-1/cPGES [74].

Because few studies have described DCs and neutrophils in the CNS, we will focus primarily on the microglia functions. It is noteworthy that although there is a close relationship between the peripheral macrophages and microglia, all of the knowledge concerning the peripheral cells cannot simply be extended to microglia cells that are inserted in a unique environment.

Initially, astrocytes were reported to be the major source of prostanoids within the CNS [75], but later studies have demonstrated that microglial cells can release higher levels of PGE_2, PGD_2, and TXB_2 than astrocytes [76]. Similar to peripheral macrophages, COX-2 is the main enzyme expressed by microglia after activation [77]. LPS induces high levels of PGE_2 synthesis by upregulating COX-2 and mPGES-1 expression [76, 78]. Additionally, activation of microglia by TLR can be modulated by further PGE_2 synthesis. Although factors such as TGF-β [79], TNF-α [80], norepinephrine [81], adenosine, and PGE_2 [82], can act as COX-2 positive regulators, other factors, such as IFN-γ [83], IL-10 [79], NO [83], and lipocortin [84] are negative regulators of COX-2 expression and activation. Interestingly,

PGE_2 synthesis is rapidly augmented when microglia are treated with phosphatidylserine (PS) liposomes in a manner that is dependent on the COX-1/mPGES-2 axis [85].

From the moment that PGE_2 is released, it acts in close proximity to its production site in an autocrine or paracrine manner. In general, PGE_2 acts as a suppressive mediator of the microglia. In the CNS, PGE_2 primarily causes enhanced levels of cAMP [80], which further suggests a role for EP_2 and EP_4 in the mediation of CNS inflammation. Supporting its suppressive functions, studies of TLR4-mediated microglial activation have shown that PGE_2 can inhibit the production of TNF-α [86] and IL-12 [87], IL-18 [88], the expression of the B7-2 (CD86) co-stimulatory molecules [89], the enhancement of IL-10 and IL-6 production, and the expression of inducible nitric oxide synthase (iNOS). Additionally, a recent study has associated PGE_2 with decreased microbicidal activity by microglial cells in meningitis [90].

In addition to its inflammatory roles, PGE_2 is related to several central functions, such as fever (thermogenesis), the neuroendocrine axis, food intake, and behavior during sickness. Circulating IL-1β acts at the blood-brain barrier (BBB) to induce COX-2 expression and PGE_2 synthesis, and PGE_2 subsequently diffuses into the brain parenchyma to perform its actions [91]. Recent studies have revealed that central COX-2 inhibition did not abrogate fever induction or the increases in plasma corticosterones and anorexia, which suggests that other sources of PGE_2, such as COX-2-dependent peripherally synthesized PGE_2 or COX-1-dependent centrally produced PGE_2 [92], are involved. Interestingly, PGE_2 production in the spinal cord is elevated by peripheral inflammation through COX-2 and mPGES-1 induction, which is correlated with peripheral edema potentiation, enhanced neuron hyperexcitability, and hyperalgesia [93]. Moreover, COX-2-dependent PGE_2 is an important signaling mediator for synaptic modification [94].

The role of PGE_2 in the brain remains controversial, and its differential effects depend on its specific receptor [95]. Because the expression and timing of the EP receptors vary according to the cell type and neuronal stimuli, the specific role of each EP receptor depends on its specific context (for an extensive review, see [96]). The EP_3 receptor is likely not associated with inflammatory roles, while the EP_2 and EP_4 receptors appear to have opposing activities [96]. Although the EP_2 receptor is related to a proinflammatory neurotoxic effect in activated microglia [97], the EP_4 receptor has an anti-inflammatory, neuroprotective role [98]. These contradictory effects reflect the differential expression and timing of the EP receptors.

Consistent with the myriad activities of PGE_2 and the dependence on the expression of specific EP receptors in different cell types, studies that investigate the roles of PGE_2 in the CNS should be addressed carefully. The inflammatory effects of PGE_2 are related to its dual neuroprotective and neurotoxic roles, and unless the PGE_2 paradoxical effects are finely tuned, neurodegenerative diseases could occur. A full understanding of the roles of PGE_2 and the dynamics of EP receptors in the CNS requires the study of the restrained areas of the CNS and the endogenous PGE_2 functions relative to the different cell types and receptors that are involved.

6. Reproductive Tract

Uterine macrophages are an important source of PGs for uterine activity [99]. They are known to be potent agonists that promote contractile activity in the uterus, and either PGs or its precursor treatments initiate preterm labor throughout gestation. Therefore, LPS-induced uterine activation may be due to increased levels of proinflammatory cytokine and PGE_2. Furthermore, exogenously added PGE_2 analogs can reduce the innate immune defenses within the reproductive tract. Slama et al. provided a good example of the role of PGE2 in inhibiting innate immune response. They injected a PGE_2 analog into the maternal cervix of cows for 1 wk following calf delivery and observed an increased purulent uterine secretions, increased frequency and severity of bacterial contamination of the uterus, and reduced levels of antibodies in uterine secretions. Pharmacological PGE_2 administration facilitated the establishment of chlamydial infections of the murine female reproductive tract [100]. We have shown that the intrauterine administration of misoprostol in rats infected with *Clostridium sordellii* further enhanced the bacterial numbers in the uterine tract and was followed by decreased animal survival. This effect was associated with the inhibition of TNF-α and defensin secretion by decidual macrophages and uterine epithelial cells [101]. Although little is known about the potential of misoprostol to suppress the reproductive tract's innate immunity, a study reported an increase in the rate of infections when misoprostol was administered orally, and the rate increased with intravaginal administration [102]. This may help to explain the connection between medical abortion and clostridial endometritis in contrast to infections that are caused by more commonly encountered pathogens.

7. Peritoneal Macrophages

Peritoneal macrophages are extensively used as a model to investigate macrophage function. This cell type is a standard model used to identify inflammatory responses, cellular metabolism, and apoptosis. Resident peritoneal macrophages exhibit low responsiveness to inflammatory stimuli relative to inflammatory peritoneal macrophages that are recruited by inflammatory stimuli, such as thioglycollate, peptone or glycogen. Resident peritoneal macrophages express mainly EP_4 but not EP_2 mRNA at basal levels. In the presence of LPS, the expression of EP_4 mRNA is downregulated to levels that are lower than in nonstimulated macrophages, and the expression of EP_2 mRNA is transiently increased after 3 h of stimulation [103].

Peritoneal macrophages have a greater capacity for PGE_2 synthesis than macrophages from different organs, such as alveolar macrophages or spleen macrophages. These cells have higher levels of cytosolic and membrane COX-1 expression in activated cells, which are similar to the levels of COX-2 expression after LPS treatment [104].

The effect of PGE_2 in the inhibition of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, was initially demonstrated in peritoneal macrophages upon TLR4 activation [105]. However, recent studies described that the effects of PGE_2 are due to the production of IL-10 [106]. However, the suppressive effect of PGE_2 on IL-6 production is controversial and seems to be dependent on the inflammatory stimulus used. In addition to the modulation of cytokines, exogenous PGE_2 can also modulate the expression of the cell surface receptors of peritoneal macrophages. The addition of different concentrations of PGE_2 induces an increase in CD14 on the surface of peritoneal macrophages through the activation of cAMP/PKA, which results in the activation of AP-1. The treatment of macrophages with a PKA inhibitor or with antisense c-fos and c-jun oligonucleotides in the presence of PGE_2 prevented the increase of CD14 on the surface of these cells [107].

PGE_2 modulates a broad range of cytokines in peritoneal macrophages involved in inflammatory processes. Endogenous PGE_2 production in LPS-stimulated resident peritoneal macrophages acts as a brake for TNF-α and IL-12 synthesis [103]. The activation of peritoneal macrophages with other macrophage activators, such as IFN-γ and the fungal particle zymosan, induces the synthesis of cytokines, chemokines, lipid mediators, and reactive nitrogen and oxygen species that directly or indirectly modulate the synthesis of PGE_2. Of the mediators that modulate PGE_2 synthesis in these cells, NO seems to play a key role in inhibiting PGE_2 biosynthesis by nitrosylating and preventing the activity of COX-2 and mPGES [108].

The capacity of PGE_2 to modulate cytokine production clearly influences the inflammatory response during injury and infection. The susceptibility or resistance to infection in different mice strains could be associated, at least in part, with the ability to stimulate the production of eicosanoids from phagocytes. When they are stimulated with LPS, peritoneal macrophages isolated from Balb/c mice produce approximately 3-fold more PGE_2 than the macrophages isolated from other mouse strains, such as C57BL. The higher levels of PGE_2 in the peritoneal macrophages of Balb/c mice are associated with high expression levels of sPLA2 type V and mPGES mRNA relative to the levels in the macrophages of C57BL mice. The increased capacity to produce PGE_2 by the macrophages isolated from Balb/c mice directly reflects the inhibition of cytokines, such as IL-12 and TNF-α [109].

The peritoneal site also represents a primary organ to generate macrophage cell lines, which are very often used to study macrophage behavior and functions. Below we will highlight some of the key human and murine cell lines used to study PGE2 production and actions.

8. RAW 264.7 Cells

RAW 264.7 cells are mouse macrophage-like cells established from the ascites of a tumor that was induced into a male Balb/c mouse by an intraperitoneal injection of Abselon leukemia virus (A-MuLV). These cells are extensively studied in models of inflammation, metabolism, and apoptosis, and they are used for *in vitro* drug screening. Currently, many reports have shown that EP_4 is the most abundant EP receptor in RAW 264.7 cells, followed by EP_2 and EP_3 but not EP_1 [110]. The expression of these receptors in RAW 246.7 cells can be modulated in a manner that is dependent on the inflammatory stimuli. TLR4 activation increases EP_2 and inhibits EP_4 receptor mRNA expression. In contrast, if these cells are stimulated only with IFN-γ, the expression of EP_2 and EP_4 decreases in a concentration-dependent manner [111].

Several inflammatory mediators, including TNF-α, IL-1 [112], and IFN-γ [113], can directly or indirectly increase the expression of COX-2 in RAW 246.7 cells. However, COX-2 expression and PGE_2 synthesis in IFN-γ-treated RAW 264.7 cells is directly regulated by TNF-α [114]. In the presence of an inflammatory stimulus, PGE_2 appears to have an autocrine effect in RAW 264.7 cells and can self-regulate the expression of COX-2. The pretreatment of cells with PGE_2 or EP_2/EP_4 agonists followed by the stimulation with LPS induced an increase in COX-2 expression, and this expression was completely inhibited in the presence of an adenylyl cyclase inhibitor [115].

9. U937

U937 is a cell line isolated from the histiocytic lymphoma of a 37-year-old male and is used to study the differentiation of monocytes into mature macrophages in the presence of different stimuli, such as IFN-γ, phorbol 12-myristate 13-acetate (PMA), and vitamin D [116]. In PMA-differentiated cells, EP_4 is the predominant receptor, while only low levels of EP_1, EP_2, and EP_3 were detected [117]. Unstimulated U937s expressed high levels of EP2 on the surface; however, when these cells were incubated with different concentrations of PMA, the expression of EP_2 and the cAMP levels that were induced by PGE_2 decreased in a manner that was dependent on PKC [118].

Undifferentiated U937 cells produce low levels of PGE_2; however, in the presence of 12-0-tetradecanoylphorbol13-acetate (TPA), these cells produce high levels of PGE_2. U937 cells express high basal levels of PLA_2, $cPLA_{2\alpha}$, and $iPLA_{2\beta}$, and the presence of IFN-γ does not alter the expression of these proteins. The activation of these cells by the aggregation of FcγRI promotes the generation of PGE_2, but only $iPLA_{2\beta}$ appears to be involved in the release of AA and the generation of this prostanoid [119]. Untreated U937 cells or differentiated U937 cells in the presence of 1,25-dihydroxyvitamin D3 express only COX-1; however, when the differentiated cells are stimulated with serum-treated zymosan (STZ), they begin to express high levels of COX-2; in the presence of exogenous AA, they produce high levels of PGE_2 [120]. U937 cells differentiated in the presence of PMA express COX-2 and high levels of PGE_2, IL-1β, and TNF-α after 6 h of stimulation with LPS. However, unlike other cell types, the increased COX-2 levels in U937 cells are independent of the presence of IL-1β and TNF-α because the treatment of these cells with the respective neutralizing antibodies does not interfere with the expression of LPS-induced COX-2 [121].

10. Therapeutic Approaches

Because PGE_2 is the major PG product of most organs and its synthesis is upregulated during inflammatory conditions, which include infections and pathophysiologic conditions, it is expected that PGE_2 plays a nonredundant role in controlling the inflammatory response and modulating phagocyte function in diverse organs. Increased plasma PGE_2 levels have been reported in murine models and in patients who have undergone bone marrow transplantation [16, 122], are infected with HIV [123], display protein-calorie malnutrition [124], are smokers, are aging [125], or have cancer [126] or cystic fibrosis [127]. In all circumstances, these conditions are associated with susceptibility to infection. More specifically, in a murine bone marrow transplantation model, high levels of PGE_2 were observed in the lung and peritoneal lavage fluid, and the overproduction of PGE_2 by multiple cell types, including AMs, PMNs, and alveolar epithelial cells, was observed [16]. Similarly, a bactericidal PMN defect in guinea pigs following thermal burn injury has been linked to increased intracellular cAMP levels and the overproduction of PGE_2 [128]. In both a murine bone marrow transplant model and also a thermal burn injury, these defects were overcome by treatment with COX inhibitors. While COX inhibition is conventionally regarded to be an "anti-inflammatory" strategy, an alternative possibility is that COX inhibitors or other nonsteroidal anti-inflammatory drugs (NSAIDs) can prevent the overproduction of immunosuppressive PGE_2, which may instead represent an "immunostimulatory" strategy. In contrast, in conditions in which PGE_2 exerts proinflammatory activities, such as in arthritis, atherosclerosis, and fever, COX inhibition is also an attractive target due to its analgesic and antipyretic properties. These drugs also have the beneficial effects of pathogen clearance. This effect has been shown that the *in vivo* treatment with NSAIDs enhances microbial clearance in different models of infection [26]. Although it has not been explicitly tested, we speculate that PGE_2 inhibition by NSAIDs should lead to reductions in intracellular cAMP levels, which may account for the immunostimulatory effects of NSAIDs in these models.

11. Conclusion

In summary, pharmacological inhibition or receptor genetic deletion in mice has unveiled the big diversity and distinct biological effects of PGE_2. Depending on cell-specific signaling programs and the context of injury, EP receptors can mediate either bad or protective effects in processes that mediate various diseases. The development of highly selective pharmacological agents that targets individual EP receptors should be studied in clinical trials in different disease settings.

Authors' Contribution

Alexandra Medeiros and Camila Peres-Buzalaf are equally contributed.

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Review Article

PGI_2 as a Regulator of Inflammatory Diseases

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Prostacyclin, or PGI_2, is an end product derived from the sequential metabolism of arachidonic acid via cyclooxygenase and PGI synthase (PGIS). The receptor for PGI_2, IP, can be found on a variety of cell types and signaling through this receptor exhibits broad physiological effects. Historically, PGI_2 has been understood to play a role in cardiovascular health, specifically having powerful vasodilatory effects via relaxation of smooth muscle and inhibiting of platelet aggregation. For these reasons, PGI_2 has a long history of use for the treatment of pulmonary arterial hypertension (PAH). Only recently, its importance as an immunomodulatory agent has been investigated. PGI_2 regulates both the innate and adaptive immune systems and its effects are, for the most part, thought to be anti-inflammatory or immunosuppressive in nature, which may have implications for its further clinical use.

1. Introduction

Prostacyclin, or PGI_2, was first reported by Needleman and Vane in 1976 and is an end product derived from the sequential metabolism of arachidonic acid via cyclooxygenase-2 (COX-2) and prostacyclin synthase (PGIS) [1]. COX-2 is expressed upon specific stimulation such as cytokines, growth factors, bacterial endotoxins, tumor promoters, and hormones by macrophages, neutrophils, and activated mesenchymal cells [2–4]. There is rare expression of COX-2 in unstimulated tissues [5–7], but it can be present at low basal levels in endothelium and the renal macula densa [2, 5]. COX-2 is typically associated with proinflammatory conditions such as atherosclerotic lesions, aortic aneurysms, or vascular damage where COX-2 derived products likely provide a protective effect [8–10]. COX-2 is inhibited by nonsteroidal anti-inflammatory (NSAIDS) and specific COX-2 inhibitors, which may have tissue specific effects.

Several additional cells types have been shown to express COX-2 and PGIS and they include fibroblasts, follicular dendritic cells, endothelial cells, smooth muscle cells, and thymic nurse cells. Production of PGI_2 is decreased by the inhibition of PGIS by tyrosine-nitrating agents such as peroxynitrite [11] and tetranitromethane [12]. Lastly, PGIS can be limited by substrate-dependent suicide inactivation if there is adequate conversion of PGH_2, the substrate for PGIS, which causes accumulation of inactivated enzyme [13].

PGI_2 is primarily produced in mammalian vasculature with elevated levels in pulmonary arterial segments when compared to systemic circulation [14]. As such, PGI_2 has been understood to play a role in cardiovascular health specifically inhibiting platelet aggregation and having powerful vasodilatory effects via relaxation of smooth muscle [5, 15]. PGI_2 analogues have been successfully used for therapy in pulmonary arterial hypertension, peripheral occlusive disease, vascular complication of diabetes mellitus, and treatment of reperfusion injury. Only recently, its importance as an immunomodulatory agent has been investigated.

2. PGI_2 Receptor Signaling

The cell surface receptor for PGI_2 is a seven transmembrane G-protein-coupled receptor termed IP [6]. IP is coupled to a guanosine nucleotide-binding α-stimulatory protein ($G\alpha_s$). When activated by PGI_2, IP stimulates adenyl cyclase leading to increased intracellular cyclic AMP (cAMP) (see Figure 1). Increased cAMP then leads to activation of protein kinase

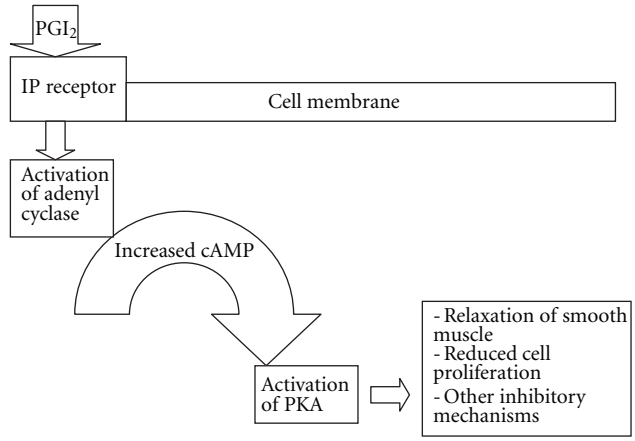

Figure 1: PGI_2 receptor signaling.

A (PKA) and further phosphorylation of key proteins [14–16]. These actions culminate in relaxation of smooth muscle, reduced cell proliferation, and other inhibitory mechanisms. IP is found on a variety of cell types and exhibits broad physiological effects. Mouse IP receptors have been identified on neurons, smooth muscle cells of the aorta, coronary arteries, pulmonary arteries, and megakaryocytes. Human IP receptors are present on multiple cell types including platelets, medullary thymocytes, neutrophils, dendritic cells, eosinophils, T regulatory cells, and activated T cells [4, 17]. IP receptors are also found on many cell types in the lung such as macrophages, pneumocytes, smooth muscle cells, and fibroblasts [17]. In addition to the single known membrane IP receptor, a peroxisome proliferator-activated nuclear receptor (PPAR) functions as a transcription factor after activation by PGI_2 [3, 15]. There are three PPAR isoforms, α, δ (β), and γ [15]. PPARγ, expressed in adipose tissue, spleen, and large intestines predominately, is thought to be downstream of the activated IP membrane receptor and can be stimulated via stable PGI_2 analogues. PPARs are responsive to not only PGI_2 but also a broad range of ligands [15].

3. PGI_2 in Specific Disease States

3.1. Allergic Inflammation. One of the areas that PGI_2 has been extensively studied is allergic inflammation. PGI_2 is produced in the human lung during allergic reactions, suggesting that it may have a regulatory role in allergen-induced inflammation [18, 19]. The *in vivo* role of PGI_2 in mediating allergic inflammation has been investigated both in IP-deficient mice, which examines the role of endogenous PGI_2 signaling, as well as exogenous administration of PGI_2. First, we will review studies that use IP-deficient mice in determining how endogenous PGI_2 modulates allergen-induced lung disease.

In an acute model of allergic inflammation induced by ovalbumin (OVA), IP-deficient mice had significantly greater airway inflammatory responses consisting of increased plasma extravasation, leukocyte accumulation, and both IL-4 and IL-5 production in the airways after sensitization and exposure to inhaled antigen [20]. In addition, the IP-deficient mice had elevated total serum IgE and antigen-specific IgE when compared to wild-type mice [20]. These findings support endogenous PGI_2 as an important suppressor of acute allergic inflammation. Similar findings were reported in a chronic model of OVA-induced lung inflammation. In this IP-deficient mouse model, there was a significant increase in the level of inflammatory leukocytes in the airway, serum OVA-specific IgE, and lung expression of T helper type 2 (Th2) cytokines such as IL-4, IL-5, and IL-13. The IP-deficient mice had more subepithelial fibrosis compared to wild-type mice, possibly related to the upregulation of collagen synthesis [21]. Therefore, inability to signal through IP led to enhanced acute and chronic allergic inflammation and airway remodeling.

In addition to IP-deficient mice, the role of COX-2 inhibitors contributes to the knowledge of how endogenous PGI_2 regulates allergic inflammation. After OVA inhalation in a DO11.10 transgenic mousemodel of T-cell-mediated airway inflammation, there was an increased level of PGI_2 [4]. Blocking PGI_2 via COX-2 inhibition resulted in a marked increase in Th2 mediated lung inflammation in response to OVA challenge. COX-2 inhibition and the prevention of PGI_2 formation was associated with increased bronchial airway hyperresponsiveness, elevated lung expression of Th2 cytokines, and decreased IL-10 production [4]. These results highlight a possible risk enhanced airway inflammation with use of specific COX-2 inhibitors in allergic asthmatics by blocking production of an immunoinhibitory prostanoid such as PGI_2.

Animal models of exogenous PGI_2 administration further supports that this prostanoid inhibits allergic inflammation. In an OVA sensitized mouse model using adoptive transfer of DO11.10 Th2 cells pretreated with PGI_2, there was significantly decreased pulmonary inflammation and airway hyperreactivity [22]. A protective effect of PGI_2 on acute airway function is further suggested by a null effect on ozone induced airway inflammation and hyperresponsiveness in mPGES-1 deficient mice. PGI_2 metabolites in the BAL of these mice were increased, while changes in other prostanoids favored deleterious effects on lung

function [23]. PGI_2 suppressed Th2 infiltration of the lung via strong inhibition of CCL17-induced chemotaxis. IP deficient Th2 cells were unaffected and migrated normally [22]. Lastly, in a mouse model of asthma, inhaled iloprost, a PGI_2 analogue, decreased the cardinal features of asthma such as Th2 cytokine production, eosinophilic airway inflammation, goblet cell hyperplasia, and bronchial airway hyperresponsiveness [24]. Iloprost inhibited the maturation and migration of antigen presenting lung myeloid dendritic cells, decreased costimulatory molecules, and decreased the induction of allergen-specific Th2 response [24]. These findings suggest a role for inhaled iloprost in the treatment of asthma.

3.2. Inflammation-Induced Anorexia. We have shown that PGI_2 signaling via the IP receptor reduces allergic inflammation. The effect of decreased appetite in acute inflammation, such as in the setting of IL-1β and lipopolysaccharide (LPS) administration, has been suggested to be PG dependent. In a mouse model, PGI_2 signaling decreased the level of circulating ghrelin, a peptide produced predominantly by the stomach, which has potent stimulatory effects on appetite [25]. This finding was very similar to the effect of LPS on circulating levels of ghrelin and suggests a role for PGI_2 in the acute sickness behavior of anorexia. IL-1β induced ghrelin expressing cells to produce PGI_2. Nonspecific inhibition of PG production via NSAIDs reversed the decrease in circulating ghrelin caused by the acute inflammatory stimulation of LPS specifically [25]. These results suggest that PGI_2 may suppress appetite in certain acute inflammatory disease states.

3.3. Liver Injury. In a mouse model of a concanavalin-A (ConA-) induced immune-mediated liver injury mimicking hepatic inflammation, beraprost, a PGI_2 analogue, decreased tissue damage [7]. COX-2 deficient mice developed more severe ConA-induced liver damage compared to wild-type mice or COX-1 deficient mice. Treatment with beraprost/ConA had a protective effect with a more than 10-fold decrease in serum alanine aminotransferase (ALT) levels compared to those treated with vehicle/ConA alone. Hepatic mRNA levels and expression of both TNF-α and IFN-γ by natural killer T cells (NKT) and T cells, key in the development of ConA induced liver disease, were decreased in the COX-2 deficient mice after treatment with beraprost when compared to vehicle/ConA alone treated mice. The protection provided by beraprost is postulated to stem from the maintenance of hepatic blood flow via beraprost's vasodilatory effects [7]. These findings suggest that PGI_2 analogues may be of benefit to patients suffering from inflammatory liver disease such as hepatitis due to viral infection, autoimmune conditions, use of certain drugs, or alcohol ingestion.

3.4. Cardiovascular Disease. It is hypothesized that early depletion of PGI_2 from endothelial tissue could lead to the pathogenesis of atherosclerosis by causing deposition of adipocyte lipid in smooth muscle cells [26]. Cellular micro RNA (miRNA) is an important known negative regulator of gene expression. PGI_2 regulated miRNA expression in a mouse adipose tissue-derived cell line leading to diminished deposition of lipid in cells [15]. The importance of this lies in the possibility of a relationship between loss of normal PGI_2 production in the setting of obesity and atherosclerosis.

Iloprost was investigated for its effects on leukocyte adherence in intestinal venules and subsequent microvascular blood flow in a rat endotoxemia model. Iloprost attenuated leukocyte adherence in both postcapillary and collecting intestinal venules and improved intestinal microvascular blood flow without affecting mean arterial pressure or heart rate [27]. These findings suggest a role for iloprost therapy to reduce endotoxin-induced intestinal injury.

Retinoic acid induces PGIS and therefore synthesis of PGI_2 in human umbilical vein endothelial cells [2]. Retinoic acid is important in the development of the cardiovascular system during embryonic development, angiogenesis and has antithrombotic and antiatherogenic qualities. 13-*cis*-retinoic acid (13-*cis*-RA) a molecule with anti-inflammatory, antitumor, and immunomodulatory effects elevated PGI_2 levels as measured by 6-oxo-$PGF_{1\alpha}$. Consistently, arachidonic acid induced platelet aggregation was inhibited by 13-*cis*-RA. Treatment with the inflammatory cytokine IL-1β alone rapidly inactivated PGIS in these same cells followed by a diminution of PGI_2. These findings support a role for 13-*cis*-RA as a possible selective treatment for patients with inflammatory cardiac disease. When IL-1β was given in combination with 13-*cis*-RA, 13-*cis*-RA was able to overcome the inhibitory effects of IL-1β, leading to increased PGIS expression and PGI_2 levels [2]. Similarly, in human vascular smooth muscle cells exposed to IL-1β, hypoxia overcame the inhibitory effects of IL-1β and increased PGI_2 production [5]. Hypoxia alone in these same cells elevated PGIS expression and PGI_2 levels. These findings suggest that hypoxia could drive an adaptive response in vascular cells and plays a role in protecting vascular cells when inflammation is present [5].

3.5. Emphysema. PGIS expression was lower in arteriolar endothelium of human emphysema lung tissue compared with normal lung [28]. Cigarette smoke extract suppressed PGIS gene expression suggesting that its decrease could lead to deleterious effects on lung vasculature in this setting. Mice exhibiting overexpression of PGIS in the pulmonary vasculature had decreased endothelial cell apoptosis after chronic tobacco smoke exposure [28]. Cigarette smoke extract may bind to CpG sites in DNA leading to disruption of transcriptional regulation suggesting a mechanism for the minimization of PGIS gene expression.

3.6. Cytokine-Mediated Inflammation. IL-1β, TGF-β, and bradykinin, in human pulmonary artery smooth muscle, decreased the production of cAMP in response to subsequent administration of PGI_2 analogues, providing data that inflammation can impair the actions of PGI_2 analogues in pulmonary hypertension treatment [14]. IL-1β, TGF-β, and bradykinin also decreased adenylyl cyclase mRNA and

increased G-α inhibitory ($G\alpha_i$) protein levels with subsequent reduction in IP mRNA expression [14]. Importantly, this could be a rational for the development of tolerance to PGI_2 analogue medications.

Cytokine toxicity is mediated in many cell types through the arachidonic acid metabolism pathway via inducible COX-2 production [3]. Proinflammatory cytokines are major effectors of programmed cell death in the development of type 1 diabetes mellitus. Using a model of human insulin producing pancreatic β cells, PGIS overexpression protected against cytokine toxicity via decreased activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and subsequent prevention of inducible nitric oxide synthase (iNOS) and decreased cytokine-induced caspase-3 activation. Therefore, low-endogenous PGIS expression may have a pathogenic part in the development of pancreatic β cell death [3].

3.7. Fibrosis. PGI_2 has regulatory affects on fibroblast proliferation. For example, in a bleomycin-induced pulmonary fibrosis mouse model, PGI_2 functioned as an antiproliferative molecule preventing an increase in fibroblasts and providing protection against loss of lung function [17]. Similarly, in an additional mouse model of bleomycin-induced pulmonary fibrosis, mice treated with iloprost had a significant reduction in airway and pulmonary parenchyma inflammatory cells and deposition of collagen with improvement in lung static compliance, tissue elastance, and overall survival [29]. The proposed mechanism for these changes is the upregulation of antifibrotic mediators such as interferon-γ (IFN-γ), the chemokine CXCL10, and the downregulation of proinflammatory and profibrotic cytokines such as TNF-α, IL-6, and TGF-β [29].

Using a mouse model of PGIS overexpression specifically in lung epithelium, mortality related to bleomycin-induced acute lung injury was decreased [30]. In addition to decreased mortality, there was a reduction in parenchymal consolidation, apoptosis of lung tissue and weight loss. These findings were explained by *in vitro* and *in vivo* PGI_2 induced expression of NADP(H) : quinone oxide reductase 1 (Nqo1), an enzyme known to inhibit the generation of reactive oxygen species and therefore protective against oxidative stress. The PGIS-overexpressing mice had elevated levels of this antioxidant prior to administration of bleomycin and afterwards [30]. Taken together, these three studies suggest that PGI_2 may provide protection against bleomycin-induced lung injury.

3.8. Viral Infection. Signaling through IP had protective effects in the setting of respiratory syncytial virus (RSV) infection in a mouse model and one study suggests it may be beneficial in human disease. During RSV infection, in a mouse model of overexpressed PGIS in bronchial epithelium, weight loss, viral replication, and IFN-γ production were all decreased compared to controls [31]. Histopathology results showed decreased pulmonary edema. In contrast, IP-deficient mice had longer more severe illness with prolonged viral replication [31].

RSV infection also elevated a urinary metabolite of PGI_2 in human infants [32] suggesting PGI_2 may modulate virally-induced illness in people. Low numbers of a genetic polymorphism in the PGIS gene with subsequent decreased urinary PGI_2 metabolite levels, described as a genetic 9-base variable-number tandem repeat (VNTR), were correlated with more severe RSV infection. Infants with greater VNTR had less severe RSV-induced infections. Therefore, an association between lower numbers of PGIS VNTR repeats and increased severity of RSV infection identifies a host genetic factor associated with RSV disease severity [32]. Both of these studies support a protective role for PGI_2 in RSV-induced illness and suggest a possible therapy for acute RSV infection.

3.9. Rheumatoid Arthritis. In contrast to the seemingly anti-inflammatory properties of PGI_2, there is still debate about its effect in the setting of specific conditions [16, 33, 34]. PGI_2 and PGE_2 have been questioned as causative factors in inflammation as levels are increased in inflammatory tissues. The synovial fluid of rheumatoid arthritis (RA) patients has rich quantities of PGI_2 but the exact role of PGI_2 in RA is not fully understood [33, 34]. In a mouse model of chronic RA, IP-deficient mice were subjected to collagen-induced arthritis and had significantly decreased clinical and histologic arthritic scores despite anticollagen antibodies and complement activation similar to wild-type mice [34]. These mice were noted to have decreased levels of IL-6 in their arthritic paws. Administration of an IP agonist elevated the inflammatory cytokine IL-6 and amplified arthritis related genes such as IL-11, VEGF, FGF-2, and RANKL, increasing inflammation in the joint. Elevated IL-6 led to proliferation of the synovium, maturation of B cells, and formation of osteoclasts, all important in the pathogenesis of RA [34]. Importantly, signaling through the IP receptor required the presence of inflammatory IL-1β for many of its effects in RA. One mechanism proposed for the effects of increased PGI_2 was the enhanced production of IL-6 via activated synovial fibroblasts in the setting of IL-1β. Lack of elevated IL-6 due to IL-1β explains why patients receiving PGI_2 agonists, for example, due to PAH, do not exhibit an increase in arthritic symptoms as a side effect. The results of this study are intriguing because PGI_2 was traditionally thought to play a role in the mediation of acute inflammation, but its role in chronic inflammation has been less studied [34].

Similarly, in an IP-deficient mouse model of collagen-antibody induced chronic inflammatory arthritis, the reduction in arthritis scores was 91% compared to a wild-type control group [33]. When a highly selective IP antagonist was used in a RA mouse model, it decreased pain similar to use of NSAIDs [33]. Therefore, it appears that signaling through the IP receptor, in the setting of RA, may increase inflammation in the joint. Currently, it is believed that both PGI_2 and PGE_2 are the primary prostaglandins involved in the inflammatory pain response in RA and this provides a rationale for the empiric use of NSAIDS and COX inhibitors for treatment of chronic arthritis.

4. PGI_2 Regulation of the Immune System

4.1. PGI_2 Regulation of the Innate Immune System. PGI_2 regulates both innate and adaptive immunity and its effects are, for the most part, anti-inflammatory or immunosuppressive in nature. PGI_2 modulates the function of dendritic cells, macrophages, monocytes, endothelial cells and eosinophils [35–41]. We will now review this data.

Dendritic cells are an important bridge between the innate and the adaptive immune system. PGI_2 analogues decreased mouse bone marrow derived dendritic cell (BMDC) maturation, function, and proinflammatory cytokine production after LPS stimulation. PGI_2 analogues also increased production of anti-inflammatory IL-10 and diminished chemokine production in mice suggesting an overall anti-inflammatory effect [42]. In a dose-dependent fashion, PGI_2 analogues, decreased secretion of TNF-α, IL-1α, IL-6, and IL-12 *in vitro*. After LPS stimulation, both iloprost and cicaprost decreased levels of costimulatory molecules CD86, CD40, and MHC class II molecules on BMDC. The BMDC had a diminished ability to activate antigen-specific CD4 T cells of DO11.10 mice and led to reduced IL-4 and IL-5 production by the CD4 cells after iloprost and cicaprost treatment [42]. When the biological activity of iloprost on human monocytes-derived dendritic cells was examined, similar findings were identified. In a dose dependent fashion, iloprost inhibited the secretion of TNF-α, IL-6, IL-8, and IL-12 by monocytes-derived dendritic cells and increase secretion of IL-10 [39].

PGI_2 analogues produced a differential pattern of regulation on alveolar versus peritoneal rat macrophages suggesting variation in immunomodulatory effects of these agents on these specific cell types. Using a well characterized FcR-mediated model of phagocytosis, activation of the IP receptor inhibited phagocytosis of IgG-opsonized targets in peritoneal macrophages to a much greater extent compared to alveolar macrophages [35]. In addition, under conditions of LPS treatment, peritoneal macrophages increased production of IL-6 after administration of iloprost or carbaprostacyclin, but alveolar macrophages did not increase production to the same degree. Iloprost and carbaprostacyclin also significantly inhibited peritoneal macrophage of bacterial killing when compared to alveolar macrophages. A postulated reason for the differences is the differential expression of IP receptors on the two macrophage cell types and different receptor binding properties of PGI_2 analogues [35].

In addition to its effects on mouse innate immune cells, PGI_2 also has important regulatory effects on human dendritic cells. Iloprost increased IL-10 expression but inhibited toll-like receptor-mediated expression of TNF-α and IFN-α in human plasmacytoid dendritic cells suggesting an overall anti-inflammatory role [36]. Iloprost may, therefore, increase the tolerogenic ability of plasmacytoid dendritic cells and could potentially be useful in asthma treatment. Human follicular dendritic cells, found in germinal centers of secondary lymphoid follicles, strongly express PGIS and are able to produce PGI_2. Application of beraprost significantly reduced T cell proliferation stimulated by anti-CD3 antibody and, therefore, production of PGI_2 in the germinal centers may be a mechanism for controlling T cell numbers [38] suggesting a reason why T cells constitute a smaller population when compared to B cells in the germinal centers. Iloprost reduced IFN-γ and IL-6 induced MCP-1, IL-8, RANTES, and TNF-α production in human monocytes [40]. STAT1 activation, critical in cardiovascular inflammation, was reduced with iloprost administration and led to decreased IFN-γ induced MCP-1 expression and therefore iloprost had an overall anti-inflammatory effect [40].

The evaluation of PGI_2 analogues on the expression of Th1 and Th2 related chemokines has also been ongoing as chemokines are known to play a role in the development of asthma [43]. Monocytes are main contributors of chemokines. Human monocytes were pretreated with iloprost and treprostinil prior to LPS stimulation and then evaluated for production of Th1-related chemokines interferon-γ-inducible protein-10 (IP-10/CXCL10) and Th2-related chemokine macrophage-derived chemokine (MDC/CCL22). The PGI_2 analogues decreased IP-10 production, but enhanced MDC. The enhancement of MDC suggests that use of PGI_2 analogues could potentially increase Th2 inflammation [43].

Lastly, in a model using human lung microvascular endothelial cells, eosinophils and the chemoattractants eotaxin and C5a, PGI_2 inhibited eosinophilic migration through the endothelial barrier by affecting their chemotaxis, adhesion, and transmigration and by strengthening the endothelial barrier [37]. Chemotaxis was limited by direct stimulation of the IP receptor on eosinophils as PGI_2 caused upregulation of cAMP leading to an increase is adenylyl cyclase despite use of chemoattractants such as eotaxin and C5a. Rapid upregulation of eotaxin-induced CD11b adhesion molecule was diminished by PGI_2 leading to decreased adhesion to fibronectin. PGI_2 also prevented eosinophil transmigration by strengthening endothelial barrier function. These findings were reversed by exposing eosinophils and endothelium to an IP antagonist [37]. These studies are important because they suggest a role for PGI_2 in the maintenance of the endothelial barrier and the prevention of allergic disease.

4.2. PGI_2 Regulation of the Adaptive Immune System. Currently, it is widely believed that signaling through the IP receptor has immunosuppressive properties. PGI_2 analogues play a key role via inhibition of Th1 and Th2 cytokine production from CD4 T cells [21, 42] although the specifics remain controversial as we will see in the discussion below. B cells are also influenced by analogues of PGI_2 and are discussed at the end of this section.

In a mouse study using PGI_2 analogues, cicaprost and iloprost, IFN-γ from Th1 cells and IL-4, L-10, and IL-13 from Th2 cells were diminished in a dose dependent manner [41]. Elevated cAMP levels and downregulation of NF-κB correlated with the inhibition of these cytokines. These findings suggest an immunosuppressive capability of PGI_2 analogues [41]. In IP-deficient mice subjected to a chronic contact hypersensitivity model showed a significantly decreased contact hypersensitivity response [44].

Table 1: Therapeutic use of PGI_2—approved agents.

Agent	Pharmacology	Indications
Epoprostenol	Synthetic salt of PGI_2	PAH, transplantation, renal dialysis, and extracorporeal circulation systems
Treprostinil	IP receptor agonist	PAH
Iloprost	IP receptor agonist	PAH

In contrast to the study above, iloprost promoted Th1 cells differentiation suggesting that PGI_2-IP-signaling promotes contact hypersensitivity. Promotion of Th1 differentiation was decreased by a PKA inhibitor suggesting mediation through the cAMP-PKA pathway [44]. These findings suggest a role for PGI_2 in the promotion of Th1 pathology, which is in opposition to findings suggesting that it has more immunosuppressive effects.

In addition to immunomodulatory effects on T cells, PGI_2 regulates B cell function. In activated B cells, beraprost increased the costimulatory molecule CD86 via the IP receptor with subsequent elevation in cAMP [45]. CD86 is important as it acts as a costimulatory molecule required for T cell activation specifically Th2 activation. Its expression is elevated on B cells in the light zone of germinal center where some Th2 cells are found. Increased T cell proliferation was noted after exposure to beraprost treated B cells [45] and suggests a role for beraprost as an adjuvant in vaccine development.

5. Therapeutic Use of PGI_2

PGI_2 has a long history of use for the treatment of pulmonary arterial hypertension (PAH) [14, 16, 28]. Regrettably, it efficacy has been less than expected possibly due to the development of tolerance. A postulated mechanism for the development of tolerance is the impaired production of PGI_2 in the setting of increased thromboxane synthesis, which is also thought to be an initial step in the pathogenesis of PAH. Nonetheless, it is one of the main therapies in severe idiopathic PAH and has been shown to improve overall survival.

Epoprostenol, treprostinil, and iloprost are approved for the treatment of PAH [46] (see Table 1). Their use in PAH has been investigated since 1980 and their initial use in long-term therapy began in 1984 in the research setting. Intravenous formulations are nonselective and can cause both pulmonary and systemic hypotension via vasodilation. Side effects shared by these medications include headache, flushing, hypotension, jaw pain with initial mastication, diarrhea, nausea, musculoskeletal pain of the legs and feet, and an erythematous blotchy skin rash [46].

Epoprostenol (Flolan), a stable freeze dried synthetic salt of PGI_2, is also used in transplantation, renal dialysis, and extracorporeal circulation systems as it is an effective inhibitor of platelet aggregation. It was the first prostanoid approved for use in the treatment in PAH by the FDA in 1995 and is currently the most commonly used PGI_2 analogue [46]. It has a very short half life of 6 minutes *in vivo*. Any interruption of its infusion can cause severe rebound pulmonary hypertension and even death. Tolerance may develop over time. Due to its unstable nature requiring a continuous intravascular infusion, its use as an antithrombotic in the general population has been limited.

Treprostinil (Remodulin) was approved for use by the FDA in 2002 and is a potent IP receptor agonist. It is administered as a continuous subcutaneous (SQ) or IV infusion or an inhaled formulation and provides a long-term survival benefit in patients with idiopathic PAH. Pain at the site of administration when given SQ has limited this form of administration. The inhaled form given 4 times per day was approved for use in 2009. Currently, an oral form of treprostinil is being investigated. Overall, treprostinil has fewer side effects when compared to epoprostenol [46] and a longer half life of about 4 hours.

Iloprost (Ventavis), a PGI_2 analogue and potent IP receptor agonist, received FDA approval in 2004. It is administered via nebulization 6–9 times per day requiring 10–15 minutes per administration. Its half life is about 20–30 minutes. Administration via inhalation limits side effects such as systemic hypotension. Inhaled iloprost can cause the development of reactive airway obstruction therefore limiting its use [46].

Cicaprost is a synthetic PGI_2 analogue which is metabolically stable and bioavailable after oral administration. It is currently used in the research setting [46].

Beraprost is a stable, orally active PGI_2 analogue, used experimentally in the treatment of primary pulmonary hypertension, peripheral occlusive disease, ischemia, reperfusion injury, and in the vascular complication of diabetes mellitus [7]. Beraprost has a high-affinity binding to the human IP receptor and a longer half life of about 1 hour [45]. Benefits of beraprost have been shown in short-term trials but it appears to have attenuated effects in longer treatment courses [46].

5.1. Potential New Therapeutic Options. ONO-1301 is a novel nonprostanoid long-acting PGI_2 agonist that is currently being used in the research setting. It has both PGI_2 activity and thromboxane synthase inhibitory activity [47]. It is unlike PGI_2 as it does not contain a five-membered ring and allylic alcohol. This property adds to its stability and allows the drug to be given two times per day as a subcutaneous injection. In mouse studies, it diminished pulmonary fibrosis associated with bleomycin intratracheal injection and improved survival rates. In addition, it decreased the total cell count, neutrophil count, thromboxane B2, and total protein level in BAL fluid and inhibit ICAM-1 and VCAM-1 adhesion molecule expression in the lung tissue

[47]. Therefore, it may be a prospective candidate in the treatment of idiopathic pulmonary fibrosis in the future.

Taken together, PGI_2 functions mostly as an immunoinhibitor molecule through multiple cell types such as dendritic cells, macrophages, and T-cells.

Abbreviations

ALT: Alanine aminotransferase
BMDC: Bone marrow dendritic cells
CCL17: Chemokine (C-C motif) ligand 17
13-*cis*-RA: 13-*cis*-retinoic acid
CD: Clusters of differentiation
ConA: Concanavalin A
CXCL10: C-X-C motif chemokine 10
cAMP: Cyclic adenosine monophosphate
COX: Cyclooxygenase
FGF-2: Fibroblast growth factor
FDA: Food and Drug Administration
$G\alpha_i$: Guanosine nucleotide-binding-α-inhibitory protein
$G\alpha_s$: Guanosine nucleotide-binding α-stimulatory protein
iNOS: Inducible nitric oxidesynthase
IgE: Immunoglobulin-E
IFN-γ: Interferon-γ
IL: Interleukin
LPS: Lipopolysaccharide
MDC: Macrophage derived chemokine
MHC: Major histocompatibility complex
mRNA: Messenger RNA
miRNA: Micro-RNA
MCP-1: Monocyte chemotactic protein-1
mPGES-1: Membrane-associated PGE synthase-1
Nqo 1: NADP(H) : Quinone oxide reductase
NKT: Natural killer T cells
NSAIDS: Nonsteroidal anti-inflammatory
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
OA: Osteoarthritis
OVA: Ovalbumin
PPAR: Peroxisome proliferators activated receptor
IP: Prostacyclin receptor
PGIS: Prostacyclin synthase
PGI_2: Prostacyclin
PG: Prostaglandin
PKA: Protein kinase A
PAH: Pulmonary arterial hypertension
RANKL: Receptor activator of nuclear factor kappa-B ligand
RANTES: Regulated upon activation, normal Tcell expressed, and secreted protein
RSV: Respiratory syncytial virus
RA: Rheumatoid arthritis
STAT: Signal transducers and activators of transcription
SQ: Subcutaneous
Th1: T helper type 1 T cells
Th2: T helper type 2 T cells
TGF-β: Transforming growth factor-β
TNF-α: Tumor necrosis factor-α
VNTR: Variable-number tandem repeat
VEGF: Vascular endothelial growth factor.

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Review Article

PGD Synthase and PGD_2 in Immune Resposne

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PGD_2 is formed from arachidonic acid by successive enzyme reactions: oxygenation of arachidonic acid to PGH_2, a common precursor of various prostanoids, catalyzed by cyclooxygenase, and isomerization of PGH_2 to PGD_2 by PGD synthases (PGDSs). PGD_2 can be either pro- or anti-inflammatory depending on disease process and etiology. The anti-inflammatory and immunomodulatory attributes of PGDS/PGD_2 provide opportunities for development of novel therapeutic approaches for resistant infections and refractory inflammatory diseases. This paper highlights the role of PGD synthases and PGD2 in immune inflammatory response.

1. Introduction

Prostaglandins (PG) are a family of structurally related eicosanoids that have regulatory roles in normal physiological as well as pathological contexts [1]. Cyclooxygenase enzymes catalyze the conversion of arachidonic acid to PGH_2, which is converted to other prostanoid species including PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane (TX) A_2 by the action of specific synthases [1–3].

The synthesis of PGD_2 from its precursor PGH_2 is catalyzed by two PGD synthases (PGDSs) [4]. Prostaglandin D2 (PGD2) is involved in a wide variety of neurophysiological functions, such as regulation of body temperature, hormone release, modulation of odor and pain responses, and regulation of the sleep-wake cycle in mammals. PGD_2 is further dehydrated to produce PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2. PGD_2 acts through two receptors (DP1 and DP2 CRTH2), whereas 15d-PGJ_2 can activate peroxisome proliferator-activated receptors or inhibit a range of proinflammatory signaling pathways, including NF-κB [1, 2, 5].

The importance of the role of PGD_2 in the pathogenesis and resolution of inflammation and innate immune response is increasingly recognized [6, 7]. However, the effect of PGD_2 on inflammation is complex because PGD_2 either promotes or suppresses inflammation depending on the inflammatory milieu. This is further complicated by the fact that PGD_2 undergoes nonenzymatic processes to generate 15d-PGJ_2, an anti-inflammatory lipid. Therefore, the net effect may depend on the rate of production of distal products of PGD_2 depending upon the disease process. Here, we review the biology and role of PGD synthases and PGD_2 in inflammation and host immune response.

2. PGD Synthases

The arachidonate cyclooxygenase pathway can generate PGD_2 by the functional linkage of a series of isoformic enzymes corresponding to phospholipase A_2, cyclooxygenase and PGDS. Prostanoid formation occurs when cyclooxygenase oxygenates arachidonate converting it to PGG_2, which is then reduced to PGH_2. PGH_2, in turn, is converted to five primary active metabolites, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, or thromboxane A_2 via distinct synthases such as PGD synthase and PGE synthase [1, 2]. Two PGD synthases have been identified, lipocalin (L-PGDS) and hematopoietic (H-PGDS)

[4, 8–10]. L-PGDS and H-PGDS are quite different from each other biochemically in terms of their amino acid sequence, tertiary structure, evolutional origin, chromosomal and cellular localization, and tissue distribution and immunologically in terms of their functional relevance [10, 11].

3. Hematopoietic PGD Synthase

Hematopoietic PGD synthase (H-PGDS) was previously known as the spleen-type PGDS [9, 12] or glutathione-(GSH-) requiring enzyme for the production of PGD_2 in the peripheral tissues [12, 13]. H-PGDS is characterized as a member of Sigma class of glutathione *S*-transferase (GST) gene family [14] that catalyze the conjugation of GSH to an electrophilic substrate. The enzyme is a homodimer and folds like other glutathione transferases. H-PGDS is localized in antigen-presenting cells and mast cells of a variety of tissues and is involved in the activation and differentiation of mast cells. It is also expressed in dendritic cells, Langerhans, and megakaryoblasts [15]. H-PGDS isomerizes PGH_2 to PGD_2 selectively and effectively, whereas other GST isozymes catalyze the conversion of PGH_2 nonselectively to produce PGD_2, PGE_2, and $PGF_{2\alpha}$. The high specificity of H-PGDS for the production of PGD_2 is attributed to the unique architecture of the catalytic pocket. The deep and wide catalytic cavity of H-PGDS is striking in comparison with the narrow shallow cavities of other GSTs. The unique 3 D architecture of the cleft leads to the putative substrate binding mode and its catalytic mechanism, responsible for the specific isomerization from PGH_2 to PGD_2 [14].

H-PGDS contributes to the production of the D and J series of prostanoids in the immune system and is involved in allergic inflammatory response. Since H-PGDS is present in mast cells, Th2 cells, and other leukocytes, it is thought to be responsible for the bulk of PGD_2 production during allergic responses [16, 17]. In mouse models of asthma and allergic disease, H-PGDS has a substantial proinflammatory effect, regulating many hallmark characteristics including eosinophilia, airway hyperreactivity, mucus production, and Th2 cytokine levels. Inhibitors of H-PGDS have shown to be protective in mouse models of allergic airway inflammation [18]. The compound, HQL-79, is characterized as a specific inhibitor of human H-PGDS and has shown to exhibit a therapeutic effect when used in animal models of allergic disease and neuroinflammation [19]. Thus, selective inhibitors of H-PGDS may prove to be more useful to suppress allergic and inflammatory reactions rather than COX-1 or COX-2 inhibitors, such as aspirin, indomethacin, and coxibs because these COX inhibitors suppress the production of all prostaglandins in comparison to H-PGDS inhibitors [20–23].

While H-PGDS is proinflammatory in allergic airway diseases, H-PGDS has shown to be protective in other models of inflammation. Trivedi et al. showed that H-PGDS negatively regulates the severity and duration of delayed type hypersensitivity responses. Their data suggests that contrary to the role of H-PGDS in driving Th2-like responses in models of asthma, HPGDS may act as an internal braking signal essential for bringing about the resolution of Th1-driven delayed type hypersensitivity reactions [24]. Rajakariar et al. using H-PGDS knockout mice showed that H-PGDS synthesizes 15d-PGJ_2 during mammalian defense responses and together with PGD_2, acting through the DP1 receptor, plays a central role in controlling the onset of acute inflammation and its resolution by balancing pro- versus anti-inflammatory cytokines. These data highlight the anti-inflammatory and proresolution properties of cyclopentanone prostaglandins, PGD2 and DP1 receptors [25].

4. Lipocalin-Type PGD Synthase

Lipocalin-type PGDS is GSH independent and is identical to beta trace protein, which was discovered in 1961 as a major protein of human cerebrospinal fluid [26–28]. Because it resembles lipophilic ligand carrier proteins it was named lipocalin-type PGD synthase. L-PGDS is a bifunctional protein, acting as a PGD_2-producing enzyme as well as an intercellular transporter of retinoids or other lipophilic molecules [29]. It is the only enzyme among the members of the lipocalin gene family that binds small lipophilic substances like retinoic acid, bilirubin, and ganglioside. Structurally it is a monomer with a β-barrel structure and a hydrophobic pocket and was initially identified as responsible for PGD_2 production in the brain [10, 19, 30]. Since then it has been shown that L-PGDS is also expressed in other tissues including the heart, kidneys [31, 32], and lungs [33, 34].

L-PGDS is secreted into various body fluids, such as CSF, plasma, seminal plasma, and urine, and functions as both a PGD_2-producing enzyme and an extracellular transporter of various lipophilic substances. The L-PGDS/β-trace concentration in human serum fluctuates with circadian rhythmicity and exhibits a nocturnal increase and is best known because of its ability to induce sleep. The role of L-PGDS in several metabolic functions has since been identified. Deletion of L-PGDS leads to accelerated glucose intolerance and induces obesity [35], nephropathy, and aortic thickening [36, 37]. In animal models of ischemia lack of L-PGDS confers susceptibility to injury in brain and heart [31, 32, 38]. L-PGDS also has an inhibitory effect on progression of lung, ovarian, and colorectal cancer and some forms of leukemia [39]. Thus, it is evident that L-PGDS has several key regulatory roles that extend beyond its function in the brain.

Similar to H-PGDS in models of allergic inflammation L-PGDS has shown to be proinflammatory. Fujitani et al. reported that L-PGDS transgenic mice exhibit strong allergic lung responses and eosinophilia [40] with enhanced allergic airway inflammation. In a model of chronic allergic dermatitis blockade of L-PGDS with an inhibitor led to significant attenuation of inflammatory response [41], which was also confirmed in CRTH2 knockout mice. The proinflammatory role of L-PGDS has also been suggested in human ulcerative colitis. Hokari et al. showed that the level of L-PGDS mRNA expression is increased in UC patients in parallel with disease activity [42]. In a diabetic rat model Ogawa et al. showed that urinary excretion of L-PGDS increased preceding diabetic nephropathy [43] and the levels of L-PGDS could predict the progression of renal injury [44]. These findings have

been independently confirmed by other investigators [45]. L-PGDS in the urine is being investigated as a diagnostic biomarker of acute kidney injury and inflammation associated with diabetes, hypertension, and drug-induced nephropathy [46]. Because L-PGDS has a smaller molecular weight than serum albumin it may be expected to appear in the urine even before albuminuria and hence prove as a more sensitive marker for early detection of renal injury.

We have studied the role of L-PGDS in LPS-induced inflammation and shown that L-PGDS is induced *in vitro* in macrophages [33, 34] and *in vivo* in the lung in response to LPS and *P. aeruginosa* [33]. Our study showed that H-PGDS was constitutively expressed *in vitro* in macrophages whereas L-PGDS is induced in a time-dependent manner in response to LPS or PA103. Similarly *in vivo* studies in mice showed that the expression of L-PGD synthase was induced in response to LPS and PA103 [33]. However, the immunomodulatory effects of L-PGDS are less well studied. In a mouse model of *P. aeruginosa* infection we have shown that L-PGDS$^{-/-}$ mice have impaired host defenses whereas overexpression of L-PGDS is protective in *P. aeruginosa*-induced pneumonia suggesting a pivotal role for L-PGDS in innate immune response [33]. These studies suggest an important role of L-PGDS in immunomodulation.

5. Prostaglandin D_2

PGs are a group of 20-carbon polyunsaturated fatty acids containing a unique 5-carbon ring structure. Prostaglandins are all produced from arachidonic acid (C20:4 fatty acid) via their common intermediate, PGH_2, and are a family of structurally related eicosanoids that not only have an important role in homeostasis but also contribute to the pathology of numerous inflammatory diseases. Each prostanoid is then produced from PGH_2 by its specific terminal PG synthase such as PGD synthase in the case of PGD_2 [13]. PGD_2 is an acidic lipid mediator derived from arachidonic acid by sequential action of cyclooxygenase and PGD_2 synthases. Both H- and L-PGD synthase enzymes may form PGD_2 *in vitro,* but it is not fully understood which PGDS enzyme predominates under varied conditions *in vivo*.

PGD_2 for a long time was considered as a minor and biologically inactive prostaglandin. In the 1980s, however, PGD_2 was found to be the most abundant prostaglandin in the brains of rats [52] and other mammals including humans [53], thus suggesting that it may have an important function in the central nervous system (CNS). The physiological functions of PGD_2 have now been extensively defined and include regulation of sleep and body temperature, olfactory function, hormone release, and nociception in the central nervous system. PGD_2 also prevents platelet aggregation and induces vasodilation and bronchoconstriction. It is released from mast cells as an allergic and inflammatory mediator [54] and is responsible for the symptoms in mastocytosis patients, such as flushing, diarrhea, tachycardia, dyspnea, and deep sleep [55].

PGD_2 is further converted to 9α, 11β-PGF_2, a stereoisomer of $PGF_{2\alpha}$, which exerts various pharmacological actions different from those induced by $PGF_{2\alpha}$. PGD_2 is also readily dehydrated *in vitro* and *in vivo* [56] to produce prostaglandins of the J series, such as PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2. 15-Deoxy-$\Delta^{12,14}$-PGJ_2 has been identified as an endogenous ligand for a nuclear receptor (peroxisome proliferator-activated receptor γ), and it promotes adipocyte differentiation [5, 6].

Prostaglandin D_2 may exert proinflammatory or anti-inflammatory effects in different biologic systems. PGD_2 has been implicated in the initiation and progression of inflammation. In mouse models of asthma and allergic disease, PGD_2 has a substantial proinflammatory effect, regulating many hallmark characteristics including eosinophilia, airway hyperreactivity, mucus production, and Th2 cytokine levels [40, 47]. Moreover, inhibition of PGD_2 synthesis and PGD_2 signaling blockade has a suppressive effect on neuroinflammation in mouse models of Krabbe's disease [48]. The injection of PGD_2 into the skin has been shown to result in erythema, edema, induration, and leukocyte infiltration [49]. PGD_2 and other vasodilator prostaglandins may also contribute to inflammation by increasing local blood flow.

In contrast to these proinflammatory effects, PGD_2 and its cyclopentenone prostaglandin derivatives also have anti-inflammatory properties, with functions in resolution of inflammation. There is considerable interest in the importance of PGD_2 and its distal products in the mediation and resolution of inflammation [3, 57]. Gilroy et al. showed that in a model of experimental pleuritis PGD_2 significantly attenuated inflammation [3]. Similarly in a model of experimental colitis COX-2-derived PGD_2, acting via the DP receptor, was shown to attenuate neutrophilic infiltration into colonic mucosa [50]. In a human model of an acute inflammatory response induced by administration of LPS, which evokes transient flu-like symptoms with pyrexia and a hemodynamic response, Song et al. have shown that tetranor PGDM increases markedly during this response and that PGJ_2 has antipyretic effects [58]. These data strongly support the anti-inflammatory effects of PGD_2.

Although several studies have investigated the role of PGD_2 in inflammation, the role of PGD_2 in host immune response has been scantly studied. An earlier study showed that PGD_2 concentration, but not the PGE_2 or IL-1β concentrations, is elevated in a time-dependent manner in the CSF of patients with African sleeping sickness, caused by *Trypanosoma brucei* [59]. These investigators have also shown that mouse astrocytes and fibroblasts in culture induce the production of PGD_2 in response to *T. brucei* [60]. Although the production of PGD2 was increased *in vitro,* the functional effects of PGD2 in this setting remain unclear. In a recent investigation Zhao et al. showed that an age-related increase in PGD_2 in mice led to diminished respiratory dendritic cell migration resulting in defects in virus-specific T-cell responses *in vivo*. They further showed that administration of PGD_2 antagonist reversed this defect resulting in migration of dendritic cells with enhancement of T-cell antivirus response with increased clearance and survival [51]. These data suggest that similar to allergic airway disease PGD_2 may have immunosuppressive effects in viral infections.

In a mouse model of *P. aeruginosa* lung infection we have shown that inhibition of COX-2 improves survival in a lethal

Table 1: Summary of PGDS and PGD_2 effects in models of inflammation.

	Model	Reference
H-PGDS		
Proinflammatory	Allergic airway inflammation (mouse model)	[18]
Anti-inflammatory	Delayed type hypersensitivity (mouse model)	[24, 25]
L-PGDS		
Pro-inflammatory	Allergic airway inflammation (mouse model)	[40]
	Chronic allergic dermatitis (mouse model)	[41]
	Human ulcerative colitis	[42]
	Diabetic nephropathy (mouse Model)	[43–45]
Anti-inflammatory	LPS-induced lung inflammation (mouse model)	[33]
Immunoprotective	*P. aeruginosa* lung inflammation (mouse model)	[33]
PGD2		
Pro-inflammatory	Asthma and allergic airway inflammation (mouse model)	[40, 47]
	Neuroinflammation/Krabbe's disease (mouse Model)	[48]
	Skin inflammation (mouse model)	[49]
Anti-inflammatory	Mouse model of pleuritis	[3]
	Mouse model of colitis	[50]
Immunosuppressive	Viral infection (mouse Model)	[51]

model of *P. aeruginosa* infection [61]. The bacterial clearance of *P. aeruginosa* was enhanced in COX-2 knockout mice whereas transgenic mice that overexpress COX-2 have an impaired bacterial clearance from the lungs [62]. Our study showed that the immunomodulatory effects of inhibition of COX-2 are related to inhibition of PGE_2. We also examined the effects of administration of PGD_2 in a model of *P. aeruginosa* lung infection. Mice that were given intratracheal PGD_2 showed an enhanced clearance of *P. aeruginosa* from the lungs [33]. These results were in agreement with our studies from L-PGDS knockout and L-PGDS overexpressing mice [33]. Recently we have investigated the mechanisms by which PGD_2 may exhibit immunomodulatory effects. We have shown that PGD_2 inhibits a key proinflammatory immunoglobulin cell surface receptor TREM-1 *in vitro* in macrophages [63]. Furthermore, we have shown that PGD_2 induces the expression of Nrf2 in a DP1 receptor-dependent manner [63]. These studies provide a new paradigm and highlight a key regulatory role of PGD_2 in innate immune response to bacterial infections.

6. Conclusions

The role of PGDS/PGD_2 in regulating inflammation in a variety of organ systems and disease process is burgeoning. The inflammatory response protects the body against infection and injury but can itself become dysregulated with deleterious consequences to the host. It is now evident that endogenous biochemical pathways such as PGDS/PGD_2 get activated during defense reactions. The effect of PGDS/PGD_2 on inflammation is complex because they can either promote or suppresses inflammation depending on the inflammatory milieu. Table 1 provides a summary of the models of different effects of PGDS/PGD_2. Interdiction of L-PGDS, PGD_2, and DP receptors provides novel therapeutic approaches to modulate inflammation and innate immune responses.

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Review Article

Role of Prostaglandins in Neuroinflammatory and Neurodegenerative Diseases

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Increasing data demonstrates that inflammation participates in the pathophysiology of neurodegenerative diseases. Among the different inflammatory mediators involved, prostaglandins play an important role. The effects induced by prostaglandins might be mediated by activation of their known receptors or by nonclassical mechanisms. In the present paper, we discuss the evidences that link prostaglandins, as well as the enzymes that produce them, to some neurological diseases.

1. Neuroinflammation and Neurodegeneration

Neuroinflammation plays a key role in the progression or resolution of pathological conditions. Inflammatory responses in the brain parenchyma have been associated with the etiopathogenesis of different neurological disorders, including central nervous system (CNS) infection, brain ischemia, multiple sclerosis, Alzheimer's disease, and Parkinson's disease [1–7]. Then, it is presently clear that neuroinflammation is a key feature shared by many neurodegenerative disorders [8, 9].

Different CNS cells, such as microglia, astrocytes, oligodendrocytes, and neurons produce a plethora of inflammatory mediators, which act either in a paracrine or an autocrine fashion, leading to an intricate cross-talk between these different cell types. Among these mediators, many studies have demonstrated that CNS cells produce prostanoids and that these mediators might contribute to the normal CNS function or to enhance the neuroinflammatory and neurodegenerative processes [10]. Herein, we review the current knowledge on the role of prostaglandins, as well as the enzymes that synthesize them, in neuroinflammatory and neurodegenerative diseases.

2. Roles of Prostaglandins in Neuroinflammation: *In Vitro* and *In Vivo* Evidences

Due to the variety of prostaglandins presently known, it is reasonable to speculate that these lipid mediators might play different roles in the CNS. Below, we describe some *in vivo* and *in vitro* data with regard to the potential role of specific prostanoids in neuroinflammation.

2.1. PGE_2. To date, three prostaglandin (PG) E synthases (PGESs) have been characterized: the microsomal PGESs (mPGES-1 and mPGES-2) and the cytosolic PGES (cPGES) [11–14]. mPGES-1 is an inducible enzyme and is expressed also in activated microglia [15, 16]. There are at least four characterized PGE_2 receptors, namely, EP1, EP2, EP3, and EP4. This prostaglandin modulates the expression of inflammatory mediators by microglial cells. For example, PGE_2 and EP agonists inhibited the expression of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) generation [17] and enhanced the expression of cyclooxygenase (COX)-2 induced by lypopolysaccharide (LPS) in cultured microglia [18]. Moreover, an EP2 agonist inhibited interleukin (IL)-1β release by cultured primary rat microglia stimulated with LPS, although no reduction of this cytokine was observed with EP1, EP3, and EP4 agonists [19].

Intraperitoneal injection of LPS increased the expression of EP4 receptors in microglial cells and in the hippocampus of mice [20]. Interestingly, activation of EP4 receptors reduced the expression of different cytokines, COX-2 and iNOS in BV-2 and primary mouse microglial cells [20].

2.2. PGD_2. PGD_2 has also been shown to be important in neuroinflammatory conditions. A 6-day infusion of LPS in the fourth cerebral ventricle of rats enhanced the PGD_2 production in the brain [21]. It has been shown that PGD_2 produced by microglia acts on DP1 receptors of astrocytes, leading to astrogliosis. Moreover, oligodendroglial apoptosis was reduced by hematopoietic prostaglandin D synthase (HPGDS) inhibitor and in HPGDS-null mice, suggesting an important effect of PGD_2 in demyelination in twitcher mice, a model of Krabbe disease [22]. Expression of DP1 and HPGDS is also increased in the brains of patients with Alzheimer's disease [23].

PGD_2 also induced apoptosis of mouse oligodendrocyte precursor (mOP) cells, what could interfere in the demyelination process that occurs in multiple sclerosis [24]. It was shown that mice deficient in lipocalin-PGDS reveal an increased number of apoptotic neurons and olygodendrocytes, suggesting a protective role of lipocalin-type PGDS in the genetic demyelinating mouse twitcher [25].

2.3. 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15d-PGJ_2). 15d-PGJ_2 is a metabolite of PGD_2 and is formed from PGD_2 by the elimination of two molecules of water. At least some effects mediated by 15d-PGJ_2 are mediated by activation of the peroxisome proliferator-activated receptors (PPARs)γ. This prostaglandin has been shown to inhibit NO and tumor necrosis factor (TNF)-α production as well as expression of major histocompatibility complex (MHC) class II in activated microglia, suggesting that this prostaglandin might be important to modulate microglia functions [26]. Similar effects, such as downregulation of iNOS and cytokines, have also been observed in astrocytes [27].

2.4. PGI_2. Few studies were carried out to investigate the role of PGI_2 in the CNS. In general, these studies suggest a neuroprotective role for PGI_2 against different stimuli. For example, enhancement of PGI_2 synthesis in neuron-glia cultures by adenoviral gene transfer of PGI synthase (PGIS) reduces the expression of different inflammatory mediators induced by LPS, such as TNF-α [28], and PGI_2 receptor ligands prevented the death of hippocampal neurons induced by high oxygen, xanthine + xanthine oxidase, or serum deprivation [29]. Interestingly, 15-deoxy-(16-m-tolyl)-17,18,19,20-tetranorisocarbacyclin methyl ester, a selective central type PGI_2 receptor ligand, reduced brain damage induced by middle cerebral artery occlusion [30].

2.5. $PGF_{2\alpha}$. In rat primary neuronal culture, hypoxia increased $PGF_{2\alpha}$ content. Importantly, previous addition of this prostaglandin to the culture medium exacerbated hypoxic injury [31]. $PGF_{2\alpha}$ reduced TNF-α in primary spinal cord cultures stimulated with LPS [32]. In a model of unilateral middle cerebral artery occlusion, knockout (KO) mice to FP, the receptor for $PGF_{2\alpha}$, have less neurological deficit and smaller infarct volumes [33]. The KO animals were also less sensitive to excitotoxicity induced by unilateral intrastriatal N-methyl-D-aspartate injection. In agreement with that, in the same model, the FP agonist latanoprost increased neurological deficit and infarct size in wildtype (WT) mice [33].

3. Roles of Prostaglandins in Neurodegenerative Diseases

As previously mentioned, there are strong evidences that inflammation contributes to etiopathogenesis of neuroinflammatory and neurodegenerative diseases. Below, we discuss the involvement of prostaglandins in these neuropathological conditions.

3.1. Multiple Sclerosis (MS). A neuroinflammatory component is very evident in the etiopathogenesis of MS. MS is an autoimmune demyelinating disorder characterized by distinct episodes of neurologic deficits attributable to white matter lesions. It is the most common of the demyelinating disorders, which affects predominantly northern Europeans. The disease becomes clinically apparent at any age, although onset in childhood or after 50 years of age is relatively rare. Women are affected twice as often as men. In most individuals with MS, the illness shows relapsing and remitting episodes of neurologic deficits. The frequency of relapses tends to decrease during the course of the disease, but there is a steady neurologic deterioration in a subset of patients [34].

Modeling clinical aspects of any human disease in rodents and cells is a big challenge in all fields of research. However, it is especially more challenging to model MS, because this is an exclusively human disease, its etiopathogenesis is unknown, and this disease is multifaceted, which occur in a relapsing-remitting manner. As the toxin-induced models of demyelination such as those induced by cuprizone, ethidium bromide and lysolecithin are important to understand demyelination and remyelination but do not resemble the human disease as efficiently as the autoimmune model (experimental autoimmune encephalomyelitis EAE), this

paper will be focused on the roles played by prostaglandins in this model because of its presumed higher predictive validity [35].

3.1.1. Phospholipase A_2 (PLA_2) and COX. There is a large body of evidence demonstrating the role played by prostanoids in the onset and progression of EAE in a wide variety of animal models as well as in *in vitro* studies. Within the last decade, some studies have demonstrated that cytosolic PLA_2 ($cPLA_2$) plays a key role in the etiopathogenesis of EAE [36–39]. There are evidences supporting distinct roles played by different isoforms of PLA_2 in the onset or progression of EAE [40]. $cPLA_2$ plays a role in the onset of EAE, calcium-independent PLA_2 in the onset and progression, and secretory type II PLA_2 in the later remission phase. Immunohistochemical labeling of $cPLA_2$ was shown in either immune or endothelial cells in the spinal cord lesions of mice with EAE induced by myelin oligodendrocyte glycoprotein (MOG). Both preemptive and therapeutic treatments with a selective $cPLA_2$ inhibitor resulted in marked reduction in the onset and progression of EAE. Accordingly, the reduced clinical score parallels with reduced spinal protein concentration of COX-2 and both gene expression and protein concentrations of dozens of inflammatory mediators, including several cytokines and chemokines which are implicated with the etiopathogenesis of EAE [36]. Moreover, selective inhibition of $cPLA_{2\alpha}$ prevents EAE and suppresses Th1 and Th17 responses [38]. $cPLA_{2\alpha}$ inhibitors diminish the ability of antigen-presenting cells to induce antigen-specific effector T-cell proliferation and inflammatory cytokine production, inhibit microglial activation, and increase oligodendrocyte survival [39]. The latter study also showed that if $cPLA_{2\alpha}$ inhibitors are administered at the peak of disease or during remission—relapsing-remitting model—, the subsequent relapse is abolished. Consistently with these pharmacological studies, a genetic study showed that $cPLA_{2\alpha}$-deficient mice are resistant to EAE [37].

COX-1 and -2 are upregulated in the CNS of animals in different EAE models [36, 38, 41]. Accordingly, different selective and nonselective inhibitors of COX isoforms induce beneficial effects in different animal models of EAE. EAE onset is delayed if diet is supplemented with acetylsalicylic acid shortly after its induction in Lewis rats [42]. Indomethacin, another non-selective COX inhibitor, attenuates the progression of EAE [43].

3.1.2. PGE_2. PGE_2 seems to be the eicosanoid which is more strongly implicated with EAE onset and progression. Bolton and colleagues investigated the CNS concentrations of PGE_2, 6-oxo-$PGF_{1\alpha}$, and $PGF_{2\alpha}$ in acute EAE-affected guinea pigs [44]. They showed that a PGE_2 concentration increase in spinal cord and cerebellum precedes EAE onset, whereas the other two prostanoids were found to peak after the observation of the first clinical signs of EAE. The behavioral syndrome associated with EAE is also preceded by increased CNS concentration of PGE_2 in mice [45]. A wide screening that examined the correlation between many arachidonic acid (AA) pathway products and EAE onset and progression showed that PGE_2 (concomitantly with its receptors EP1, EP2, and EP4) is synthesized more markedly than other eicosanoids [46], suggesting an important role in exacerbating EAE. However, dual roles played by PGE_2 have been recently shown in mouse EAE. PGE_2 exacerbates Th1 and Th2 responses via EP2 and EP4 receptors during mouse EAE onset and protects the brain from immune cell infiltration via EP4 receptor [47].

mPGES-1 upregulation occurs in microglia/macrophages in the spinal cord lesions of mice with EAE induced by MOG as well as in brain tissues from MS patients. mPGES-1-deficient mice exhibit a better clinical score and suppressed Th1 and Th17 responses when compared with those of nongenetically modified control mice after EAE induction [46]. Regarding the untoward gastric and cardiovascular effects induced by COX inhibitors [48], there is an eagerness to discover compounds that target mPGES-1 for treating inflammatory diseases [49–51] because this enzyme is downstream to COX-2 in AA pathway.

3.1.3. 15d-PGJ_2. Systemic treatment with 15d-PGJ_2 inhibits EAE progression in mice, and this is associated with reduced demyelination, neuroinflammation, IL-12 production by macrophage/microglial cells, T-cell proliferation, and IL-12-induced T-cell responses [52]. Moreover, pretreatment with this agonist of PPARγ delays the onset of EAE and reduces the spinal cord infiltration of $CD4^+$ T cells and macrophages [53]. 15d-PGJ_2 suppresses the production of cytokines and/or chemokines in cultured T cells, microglia, and astrocytes [53–55]. Providing further support to the role played by 15d-PGJ_2 in EAE etiopathogenesis, it was shown that PPARγ antagonists reverse the inhibition of EAE clinical signs and Th1 response by this cyclopentanone prostaglandin [56].

3.1.4. Other Prostaglandins. As there is a correlation between increased spinal PGDS concentration and the initiation of relapsing phase of EAE, it has been suggested a role played by this isomerase in this phenomenon [57]. Indeed, PGD_2 is released from mast cells in allergic reactions, and it is suggested to modulate allergic inflammation [58, 59]. On the other hand, a more recent study showed that PGD_2, PGI_2 and 5-lipoxygenase pathways are suppressed in the acute phase of EAE and returns to constitutive levels in the chronic phase [46]. However, in a relapsing-remitting model, PGD_2 remained unaffected throughout all phases [41].

3.2. Alzheimer's Disease (AD). The first evidences supporting a role played by inflammation on AD onset rose up in the late 1980s, when many signs of inflammation in *postmortem* brains from AD patients were observed, such as activated lymphocytes and microglial cells in plaque and tangle lesions, presence of complement proteins, cell lysis, and opsonisation of debris [60–64].

3.2.1. PLA_2 and COX. It was hypothesized that the long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) could reduce the risk for AD or delay disease onset. Indeed,

McGeer et al. [65] observed a clear negative correlation between the prevalence of AD in general population versus that in rheumatoid arthritis patients taking NSAIDs, mainly salicylates. Reinforcing this evidence, a clinical trial conducted shortly afterwards, showed that treatment with indomethacin, a nonselective COX inhibitor, improves cognitive deficits in AD patients [66]. Since then, epidemiological studies have been showing either beneficial or detrimental effects induced by COX inhibitors on AD risk and delay of onset, though beneficial effects are mostly observed [67]. Despite controversy, these studies clearly show that prostanoids play an important role in AD etiopathogenesis.

$cPLA_2$, which cleaves AA from cellular membrane phospholipids, is elevated in AD brain [68]. The cyclooxygenation and subsequent isomerization of AA produces prostaglandins, which regulate immune responses and neurotransmission [69, 70]. Accordingly, increased expression of COX-1 and -2 is observed in AD-affected brains [71, 72]. One of the most versatile products of this cascade is PGE_2, which is produced by glial cells and neurons.

3.2.2. PGE_2. An increased expression of mPGES-1 and mPGES-2 is observed in the brain of AD brains [73, 74]. Moreover, patients with probable AD have higher cerebrospinal fluid (CSF) concentrations of PGE_2 than age-matched control subjects [75]. It has been shown that PGE_2 increases amyloid precursor protein (APP) gene expression and production *in vitro* [76–78]. This effect is inhibited by immunosuppressants in astrocytes [77] and is associated with EP2 receptor activation in microglial cells [78]. On the other hand, there is evidence supporting an anti-inflammatory role played by PGE_2 mediated by EP4 receptor in LPS-stimulated cultured microglial cells [20]. However, PGE_2 increases APP production via both EP2 and EP4 receptors (but not via EP1 and EP3 ones) both *in vitro* and *in vivo* [76, 79]. Hoshino et al. [76] showed that PGE_2-dependent internalization of EP4 receptor increases γ-secretase activity, which in turn leads to higher proteolysis of APP.

In transgenic mice overexpressing APP, selective inhibition of COX-2 blocks amyloid β (Aβ)-induced suppression of hippocampal long-term potentiation (LTP) and memory function independently of reductions in Aβ42 and inflammatory cytokines, but markedly dependent on PGE_2 concentrations, showing an additional mechanism by which NSAIDs may protect against AD progression and an important synaptic role of PGE_2 in this setting [80]. EP2 receptors are important mediators of PGE_2 actions on electrophysiological properties of hippocampal neurons, as $EP^{-/-}$ mice exhibit cognitive deficits in social memory tests associated with a deficit in long-term depression in hippocampus [81]. Pharmacological studies corroborate these previously mentioned findings. Either exogenous or endogenous PGE_2, but not exogenously applied PGD_2 or $PGF_{2\alpha}$, regulates hippocampal neuronal plasticity [69, 70].

3.2.3. PGD_2 and 15d-PGJ_2. One of the first studies which assessed prostaglandins concentrations in *postmortem* cerebral cortices of probable AD patients showed that only PGD_2 was increased in comparison with age-matched control subjects [82]. Indeed, PGDS expression was found to be localized in microglial cells surrounding senile plaques, and DP1 receptor expression was observed in microglial cells and astrocytes within senile plaques in human AD brains. In Tg2576 transgenic mice—a model of AD disease—, the DP1 receptor expression increases in parallel with Aβ deposition [23].

As 15d-PGJ_2 induces neuronal apoptosis [83], it was initially suggested that this prostanoid is associated with neurodegeneration. However, it was shown afterwards that 15d-PGJ_2 reduces microglial production of NO, IL-6, and TNF-α induced by Aβ40, which suggests anti-inflammatory indirect neuroprotective effect [84]. Accordingly, not only 15d-PGJ_2, but also troglitazone and ciglitazone, other compounds known to activate PPARγ and attenuate the Aβ-induced impairment of hippocampal LTP *in vitro*, supporting a possible beneficial effect on AD progression.

3.3. Parkinson's Disease (PD). PD is the second most common neurodegenerative disease, characterized by abnormal motor symptoms such as stiffness, postural instability, slowness of movement, resting tremor, and bradykinesia. The neuropathological features of PD are progressive death of dopaminergic neurons in the substantia nigra (SN) pars compacta that project to the striatum. The exact cause of this cell death is not clear, but recent studies have shown that the process may involve inflammatory reactions, in addition to oxidative stress, mitochondrial dysfunction, neural excitotoxicity, and insufficient neurotrophic factors [85–87].

It is known that, in the SN of PD brains, microglia is activated [5], and its activation has been strongly associated with CNS pathology of PD, by production of proinflammatory and cytotoxic factors, such as cytokines, chemokines, NO, reactive oxygen species (ROS), and AA metabolites [88, 89].

3.3.1. PLA_2 and COX. It has been shown that mice carrying a mutation of the $cPLA_2$ gene, leading to an absence of $cPLA_2$ activity, are resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a precursor to 1-methyl-4-phenylpyridinium (MPP^+) -induced neurotoxicity, a well-known model of PD [90]. Dopamine receptor activation also increased $cPLA_2$ activity in a rat model of PD [91].

Many data demonstrated also an alteration of COX-2 expression in PD. In fact, different studies have shown an upregulation of COX-2 in animal models of PD [92–94]. COX-2 increased expression has been also demonstrated in the SN of *postmortem* PD specimens in comparison to normal controls [95, 96]. Moreover, it has been shown that COX inhibition [93, 94, 97, 98] and COX transgenic ablation [99–101] in *in vivo* models of PD increased survival of dopaminergic neurons. However, this effect was not observed in all studies. Rofecoxib, a COX-2 inhibitor, did not change MPTP-induced neurodegeneration and, paradoxically, caused a significantly augmented basal prostaglandin production [92].

Regular use of NSAIDs is associated with a lower risk of PD compared with nonregular users of these drugs [85, 102]. However, this is still controversial, since recent studies could not demonstrate a protective effect of NSAIDs in PD [103–105]. Considering that these drugs might have other mechanisms of action unrelated to COX inhibition, it is important to evaluate the effect of specific compounds in the prevention or treatment of PD.

3.3.2. PGE_2. It has been observed that PGE_2 is significantly elevated in the CSF and SN of PD patients in comparison to control subjects. Moreover, incubation of slices of SN with AA induced an increased production of PGE_2 synthesis, suggesting an enhancement of the enzymes responsible to its production [106].

Release of aggregated α-synuclein, a major component of Lewy bodies in PD, after neuronal damage, may activate microglia. This activation could, in turn, lead to production of proinflammatory mediators, such as PGE_2 [107], contributing to the progression of nigral neurodegeneration. A pretreatment of primary mesencephalic neuron-glia mouse cultures with α-synuclein enhances the production of PGE_2. Apparently, phagocytosis of α-synuclein activates NADPH oxidase, which produces ROS, and has a crucial role in microglial activation and associated neurotoxicity [107].

In primary mesencephalic mixed neuron-microglia cultures, MPP^+, a neurotoxin that causes dopaminergic neuronal death, induced PGE_2 production. However, this effect was not observed in enriched microglia and enriched neuron cultures, indicating that is necessary an interaction between microglia and neurons for MPP^+-induced increase of the PGE_2 production, probably due to COX-2 activity. Moreover, PGE_2 was not enhanced neither in enriched astroglia nor in neuron-astroglia cultures [94]. Conversely, PGE_2 was significantly reduced in the hippocampus, striatum, and cortex of animals injected with 6-hydroxydopamine (6-OHDA) [108].

It has been shown that EP receptors are expressed differently in the SN. To date, in the rat, EP1 is restricted to dopaminergic neurons, while EP3 is expressed exclusively by nondopaminergic cells. On the other hand, EP2 is localized to both dopaminergic and nondopaminergic cells [109]. In rats, EP1, but not EP2 and EP3 receptor antagonists, reduced the dopaminergic neuronal death induced by 6-OHDA, suggesting an important effect of EP1 receptor in the neurotoxicity induced by PGE_2 [109]. Also, culture of dopaminergic neurons displayed EP2 receptors after 6-OHDA neurotoxicity, and butaprost, a selective EP2 agonist, significantly increased survival of tyrosine hydroxylase positive cells, suggesting a possible neuroprotective role of EP2 of activation [110].

Interestingly, in comparison to microglia obtained of WT animals, microglia of EP2 KO mice reveal an enhanced capacity to clear aggregated α-synuclein in human mesocortex tissue of patients with Lewy body disease. Moreover, $EP2^{-/-}$ mice were more resistant to neurotoxicity induced by MPTP, an effect that is associated with attenuated formation of aggregated α-synuclein in the SN and striatum [111].

3.3.3. PGD_2, PGJ_2, and Other Prostaglandins. PGJ_2 and its metabolites might alter the process of protein folding and aggregation, contributing to the development of PD. In human neuroblastoma SK-N-SH cells, PGJ_2 disrupts the structural integrity of microtubules and actin filaments [112]. *In vitro*, this molecule also hindered the polymerization of highly purified tubulin from bovine brain [113]. Interestingly, in cells treated with PGJ_2, microtubule/endoplasmatic reticulum collapse coincides with the formation of protein aggregates, such as ubiquitinated proteins and α-synuclein [113].

In mouse and human neuroblastoma cells, as well as in rat primary embryonic mesencephalic cultures, PGA_1, PGD_2, PGJ_2, and its metabolite Δ^{12}-PGJ_2 induced accumulation of ubiquitinated proteins and cell death [114]. PGE_2 only exhibited neurotoxic effects at high concentrations. The ubiquitination induced by Δ^{12}-PGJ_2 might be due to inhibition of ubiquitin C-terminal hydrolase (UCH) L3 and UCH-L1, implicating in an alteration of deubiquitinating enzymes, possibly contributing to the accumulation and aggregation of ubiquitinated proteins, what leads to inflammation associated with the neurodegenerative process [114]. Modification of UCH-L1, an enzyme that functions predominantly during monoubiquitin recycling in the ubiquitin-proteosome system, by cyclopentenone prostaglandins, induced unfolding and aggregation of the protein. Therefore, the deleterious effect of COX-2 in PD could be due to the production of cyclopentenone prostaglandins [115].

In addition to that, PGA_1 has been shown to reduce nuclear factor kappa B translocation to the nucleus, caspase 3 activation, and apoptosis of human dopaminergic SH-SY5Y cells induced by rotenone [116].

3.4. Amyotrophic Lateral Sclerosis (ALS). ALS is a progressive neurodegenerative condition characterized by the selective death of motor neurons [117]. This neuropathological condition can be classified as familial, in which mutations in the enzyme superoxide dismutase-1 (SOD1) can occur, or as sporadic, which encompasses 90% of ALS patients [118]. Neuroinflammation seems to play an important role in the progress of this disorder. In ALS, microglia activation and proliferation is observed in regions where there is neuron loss, like motor cortex, motor nuclei of the brainstem and corticospinal tract. Microglia might be essential for the motor neuron toxicity [119].

3.4.1. PLA_2 and COX. It has been shown that $cPLA_2$ is expressed in astrocytes and motor neurons of the spinal cord of transgenic mice carrying the gene encoding a mutant form of human SOD1 [120, 121]. In agreement with that, $cPLA_2$ immunoreactivity was also observed in the spinal cord of human SOD1-mutated familial ALS and in sporadic ALS patients [120, 122].

An increase in COX-2 expression is observed in the spinal cord of $SOD1^{G93A}$ transgenic mice [123, 124] and human cases of ALS [125, 126]. *Postmortem* examination of the ventral horn of the spinal cord of sporadic ALS patients

revealed that COX-2 immunoreactivity was increased in motor and interneurons, as well as in glia, in comparison with non-ALS controls [127]. On the other hand, COX-1 expression was detected in microglia, but not in neurons, of ALS and controls tissues, albeit no difference was observed between the two groups of patients [127].

Few attempts have also been made to elucidate the effect induced by COX inhibitors in models of ALS. In organotypic spinal cord cultures, the COX-2 selective inhibitor SC236 significantly reduced the excitotoxic damage of motor neurons induced by threo-hydroxyaspartate, a compound that inhibits astroglial transport of glutamate [128]. Therefore, it is possible that COX-2 might be involved in the excitotoxicity induced by glutamate.

Moreover, *in vivo* studies also suggested that COX might be a potential target for ALS treatment. It has been shown that traditional NSAIDs and COX-2 inhibitors reduced different pathological features developed by $SOD1^{G93A}$ transgenic mice, such as loss of motor neurons and glial activation in the spinal cord, motor impairment and weight loss, as well as these compounds prolonged the survival of the animals [120, 129–131]. Considering these evidences, Minghetti [132] suggested that COX-2 enhancement could be deleterious in ALS not only due to the enhancement of glutamate release by PGE_2 [133], but also because of the ROS produced by COX peroxidase activity.

On the other hand, Almer et al. [134] have shown a drastically reduced PGE_2 production in the spinal cord of transgenic $SOD1^{G93A}/COX\text{-}1^{-/-}$ mice, suggesting a minor role for COX-2 in the production of PGE_2 in the disease. Moreover, deficiency of COX-1 did not affect motor neuron loss and survival of the animals [134]. These results challenge the concept that COX-2 is the main enzyme involved in ALS.

3.4.2. PGE_2 and 15d-PGJ_2. PGE_2 is elevated in the spinal cord of $SOD1^{G93A}$ mice [130] and in the serum and CSF of ALS patients [127, 135], though the levels of this prostaglandin did not correlate with clinical state of the patients [135].

The role of PGE_2 was further investigated in *in vitro* models of ALS. In an organotypic spinal cord slice model, motor neuronal death induced by D, L-threo-hydroxyaspartate is reduced by PGE_2, as well as butaprost and sulprostone, EP2 and EP3 receptor agonists, respectively [136]. Interestingly, in the same study, SC58236, a COX-2 inhibitor, also reduced motor neuron loss.

EP2 receptor expression is increased in astrocytes and microglia of $SOD1^{G93A}$ mice and in astrocytes of human ALS spinal cord. Deficiency of EP2 receptor in $SOD1^{G93A}$ mice increased the survival and grip strength in comparison with $SOD1^{G93A}/EP2^{+/+}$ and $SOD1^{G93A}/EP2^{+/-}$ mice. The absence of EP2 receptor also reduced the production of different inflammatory mediators in this animal model of ALS [124].

Recently, it has been shown that mPGES-1 is enhanced in the spinal cord of $SOD1^{G93A}$ in comparison with WT mice. Interestingly, AAD-2004, a molecule that inhibits mPGES-1 and free radical formation, reduced microglia activation and motor neuron loss, as well as it improved motor function and increased survival [137].

15d-PGJ_2 immunoreactivity is increased not only in motor neurons, but also in astrocytes and reactive microglia in the spinal cord of ALS patients [138].

3.5. Huntington's Disease (HD). HD is a progressive neurodegenerative disease that reveals movement disorders and dementia as main features. This pathological condition is an autosomal-dominant pathological condition disease [139, 140]. Although there are evidences that neuroinflammation is present in HD, it is not known whether it contributes to the etiopathogenesis of the disease or whether it is solely an epiphenomenon [141].

It has been shown that in R6/2 mice, an animal model of HD, the number of microglia is reduced in some brain regions in comparison with their WT littermates. Microglia of animals at 14.5 weeks of age were also smaller in size than the same cells in the animals at 7 weeks of age, and they also revealed condensed nucleus and fragmentation of the cytoplasm within processes, suggesting an impaired function of these cells in this pathological condition [142]. On the other hand, activated microglia are present in the neostriatum, cortex, and globus pallidus of HD brains. Importantly, the reactive microglia appeared in association with pyramidal neurons presenting huntingtin-positive intranuclear inclusions [143]. Although a causal link between neuroinflammation and HD onset or progression has not been demonstrated, it is reasonable to assume that microglia might play a role in its development.

3.5.1. COX. Although there are different genetic models of HD, some compounds such as 3-nitropropionic acid (3-NP) and quinolinic acid (QA) are also used to induce striatal neuron toxicity, being therefore considered HD animal models [144–146]. COX-2 immunoreactivity is enhanced in striatal tissues 12 h after treatment of animals with QA. This enhancement was observed predominantly in neurons and microglia [147].

Chronic treatment with different COX inhibitors, such as rofecoxib, celecoxib, nimesulide, and meloxicam improved spontaneous locomotor activity and the motor performance, as well as these medicines reduced biochemical and mitochondrial alteration induced by QA [148–150]. Naproxen and valdecoxib, two COX inhibitors, also reduced 3-NP-induced motor and cognitive impairment [151]. This study suggested that these effects could be due to a reduction in the oxidative stress induced by the drugs.

Although beneficial effects were observed induced by COX inhibitors in drug-induced models of HD, similar effects are not observed in transgenic mice. For example, administration of acetylsalicylate from weaning did not induce any alteration of rotarod performance and ventricle enlargement N171-82Q mice in comparison with untreated animals. Rofecoxib also did not change motor performance and lifespan of R6/2 mice [152]. On the other hand, acetylsalicylate and celecoxib shortened life expectancy of R6/2 and N171-82Q mice, respectively [152, 153].

3.5.2. PGE_2, $PGF_{2\alpha}$, and PGA_1. Administration of 3-NP enhances PGE_2 and $PGF_{2\alpha}$ in the striatum [154, 155]. These prostaglandins are reduced by licofelone, a competitive inhibitor of COX-1, COX-2, and 5-LOX isoenzymes. In addition, this compound reduced the impairment in locomotor activity and motor performance, as well as it reduced apoptotic markers [155]. Expression of COX-2, as well as PGE_2 production, is increased in the ipsilateral side compared with the contralateral vehicle-injected side in the striatum and cortex of rats by unilateral intrastriatal injection of QA [156]. Moreover, it has also been shown that QA injection induced EP3-positive striatal neuronal loss, whereas activated microglia expressed EP3 *in vivo* after excitotoxicity injury [157].

A role for PGA_1 has also been suggested. This prostaglandin attenuated DNA fragmentation and neuronal loss and increased dopamine D1 receptor expression induced by QA in the striatum it also reduced the QA-induced activation of nuclear factor kappa B, but not activator protein-1, in this brain region [158].

4. Discussion

There is an intricate relationship between neuroinflammation and neurodegeneration. In general, acute inflammation in the CNS is triggered by a neuronal injury or infection and is short-lived. This acute response is believed to have protective aspects, since it could avoid further injury and induce tissue repair [159]. Although an acute stimulus may trigger, for example, oxidative stress, this short-term event would not interfere with long-term neuronal survival [160]. It is known that moderate microglia activation might induce neuroprotective effects, such as to scavenger neurotoxins, remove cell debris and secrete mediators which are important for neuronal survival [160]. Acute activation of these cells is a normal response to injuries, and it contributes to wound healing [161].

On the other hand, chronic neuroinflammation persists for a long time after the initial insult and normally is self-perpetuating [160]. This condition induces neuronal death, and the molecules released by the dead neurons can further activate microglia, which enhances cell death. This vicious cycle, together with the continuous production of factors that activate microglia, contributes to the chronicity of this process.

Again, microglia might play an important role in this long-term process. Intense activation and accumulation of these cells at the site of injury can induce neuronal damage, since they release a variety of neurotoxic substances. For example, the Aβ protein, which is involved in AD, can activate microglia and lead them to release neurotoxic factors such as NO, TNF-α, and superoxide, leading to the progression of this disorder [162]. An interesting finding is that a chronic inflammation induced by the infusion of LPS (a substance that strongly activates microglia) in the brain of rats resembles different features observed in AD patients [163].

Actually, it is presently not clear why the neuronal or glial cells cannot prevent the chronicity of the inflammatory process. However, it might be due to a plethora of effects. Abnormal synthesis of some proteins by neurons could continuously activate microglia, leading them to the release of neurotoxic factors. Moreover, oxidative stress is another important event that contributes to the neuronal damage observed in chronic neuroinflammation [164]. It is also possible that the senescence of immune system in the CNS could contribute to chronicity of this process. For example, it has been shown that microglia from old transgenic PS1-APP mice release an increased amount of inflammatory mediators and do not phagocytose Aβ properly in comparison to microglia from young mice [165]. Therefore, microglia senescence could play a role in the development of some neurodegenerative conditions [161, 166]. Despite these facts, the adaptive immune system might also play a role, as it has been shown that it is involved in the etiopathogenesis of PD [167].

In this context, one might assume that the production of lipid mediators, such as prostaglandins, might differently modulate neuroinflammation and neurodegeneration. Considering the roles of prostaglandins and depending on the stage of inflammation, as well as different microenvironments generated by a variety of substances, these lipid mediators could determine the survival or death of neurons.

5. Conclusion

Here we summarized the evidences that prostaglandins might play a key role in the etiopathogenesis of neuroinflammatory and neurodegenerative diseases. Prostaglandins have a plethora of actions in CNS cells that differently affect the progress of inflammation and neuronal death or survival. Therefore, inhibition of the production of a specific prostanoid or its action on its receptor would be a better mechanism to control some pathological processes. On the other hand, inhibiting the effects of some prostaglandins could also be deleterious. Thus, further studies are important to make a more complete idea the role of these lipid mediators in neuroinflammation and neurodegeneration. This knowledge might serve to develop pharmacological strategies for the treatment of neurological diseases.

Abbreviations

15d-PGJ_2: 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
3-NP: 3-nitropropionic acid
6-OHDA: 6-hydroxydopamine
AA: Arachidonic acid
AD: Alzheimer's disease
ALS: Amyotrophic lateral sclerosis
Aβ: Amyloid β
APP: Amyloid precursor protein
CNS: Central nervous system
COX: Cyclooxygenase
CSF: Cerebrospinal fluid
EAE: Experimental autoimmune encephalomyelitis
HD: Huntington's disease

HPGDS: Hematopoietic prostaglandin D synthase
IL: Interleukin
iNOS: Inducible nitric oxide synthase
KO: Knockout
LPS: Lypopolysaccharide
LTP: Long-term potentiation
MHC: Major histocompatibility complex
MOG: Myelin oligodendrocyte glycoprotein
mOP: Mouse oligodendrocyte precursor
mPGES: Microsomal PGE synthase
MPP^+: 1-methyl-4-phenylpyridinium
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS: Multiple sclerosis
NO: Nitric oxide
NSAIDs: Nonsteroidal anti-inflammatory drugs
PD: Parkinson's disease
PG: Prostaglandin
PGIS: PGI synthase
PLA_2: Phospholipase A_2
PPARs: Peroxisome proliferator-activated receptors
QA: Quinolinic acid
ROS: Reactive oxygen species
SN: Substantia nigra
SOD1: Superoxide dismutase-1
TNF: Tumor necrosis factor
UCH: Ubiquitin C-terminal hydrolase
WT: Wildtype.

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Review Article

Eicosanoids and Respiratory Viral Infection: Coordinators of Inflammation and Potential Therapeutic Targets

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Viruses are frequent causes of respiratory infection, and viral respiratory infections are significant causes of hospitalization, morbidity, and sometimes mortality in a variety of patient populations. Lung inflammation induced by infection with common respiratory pathogens such as influenza and respiratory syncytial virus is accompanied by increased lung production of prostaglandins and leukotrienes, lipid mediators with a wide range of effects on host immune function. Deficiency or pharmacologic inhibition of prostaglandin and leukotriene production often results in a dampened inflammatory response to acute infection with a respiratory virus. These mediators may, therefore, serve as appealing therapeutic targets for disease caused by respiratory viral infection.

1. Respiratory Viruses

Viruses are the most frequent cause of respiratory infection in humans. It has been estimated that viruses cause up to 90% of lower respiratory infection (LRI) hospitalizations in children less than 5 years of age and up to 40% of hospitalizations in children age 5–18 years [1]. Among the most common causes of viral respiratory infection in children and adults are respiratory syncytial virus (RSV), influenza, rhinovirus (RV), adenovirus, parainfluenza virus (PIV), and human metapneumovirus (hMPV) [2]. Viral respiratory infection also causes substantial disease burden in the elderly and immunocompromised populations [3, 4].

The host immune system faces the task of effectively clearing a virus while limiting local tissue damage and inflammation. The immune response to viruses can be protective, aiding with clearance of virus from the lungs and resolution of disease caused by viral replication. Disease associated with respiratory viruses can also be caused by immune-mediated pathology. Virus-induced inflammation can be detrimental to the host, causing symptoms during acute infection and leading to damage that contributes to long-term residual lung disease. Eicosanoids are potent lipid mediators that play a role in many biological processes, including inflammation and immune function. Two classes of eicosanoids, the prostaglandins (PGs) and leukotrienes (LTs), have been increasingly studied in the context of respiratory viral infection. Because of these effects, eicosanoids are likely to make significant contributions to the pathogenesis of respiratory virus infection.

2. Eicosanoid Synthesis

2.1. Prostaglandins. PGs are generated when phospholipase A_2 (PLA_2) releases arachidonic acid (AA) from membrane glycerophospholipids (Figure 1). Released AA is oxidized to the intermediate prostaglandin H_2 (PGH_2) by cyclooxygenase (COX). COX exists in three isoforms. COX-1 is generally constitutively expressed, while COX-2 expression is rapidly induced by growth factors and cytokines [5]. COX-3 is a recently discovered isoform whose biological role, if any, remains poorly understood [6, 7]. Once formed, PGH_2 can be converted by specific synthases to thromboxane A_2 (TXA_2), PGD_2, PGE_2, PGF_2, and PGI_2. As described below, PGE_2 has multiple effects on host immune function. PGE_2 is transported from the cell by multidrug resistance protein

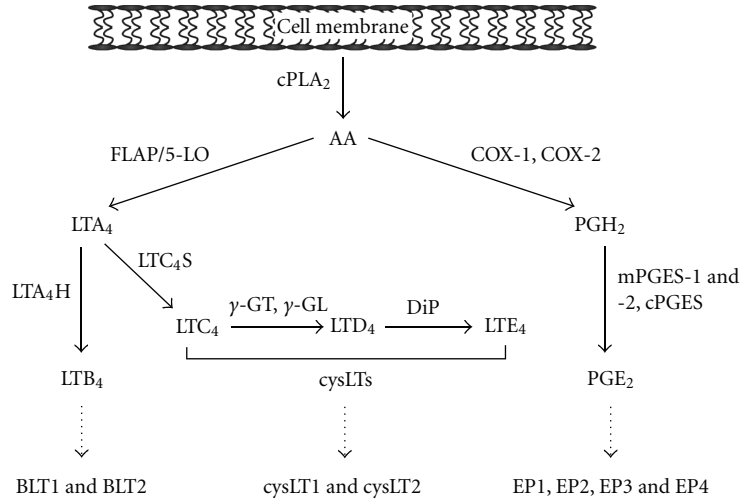

Figure 1: Synthesis of PGE_2 and the leukotrienes. $cPLA_2$—cytosolic phospholipase A_2, AA—arachidonic acid, FLAP—5-lipoxygenase activating protein, 5-LO—5-lipoxygenase, LTA_4—leukotriene A_4, LTA_4H—leukotriene A_4 hydrolase, LTB_4— leukotriene B_4, BLT1 and BLT2—B leukotriene receptor 1 and 2, LTC_4S—leukotriene C_4 synthase, LTC_4—leukotriene C_4, γ-GT-γ-glutamyl transpeptidase, γ-GL-γ-glutamyl leukotrienease, LTD_4—leukotriene D_4, DiP—dipeptidase, LTE_4—leukotriene E_4, cysLTs—cysteinyl leukotrienes, cysLT1 and cysLT2—cysteinyl leukotriene receptor 1 and 2, COX-1 and COX-2—cyclooxygenase 1 and 2, PGH_2—prostaglandin H_2, mPGES-1 and -2—microsomal prostaglandin E synthase-1 and -2, PGE_2—prostaglandin E_2, EP1-4—E prostanoid receptors 1–4.

(MRP) 4 and possibly by other unknown transporters [8]. The effects of PGE_2 are mediated by its signaling through four distinct G protein-coupled E prostanoid (EP) receptors, EP1-4. The EP1 receptor is coupled to an unidentified G protein and mediates PGE_2-induced increases in intracellular Ca^{2+} [9]. The EP2 and EP4 receptors mediate increases in cyclic AMP (cAMP) concentration by coupling to $G\alpha_s$. Four isoforms of the EP3 receptor are coupled to different G proteins, although the major EP3 receptor signaling pathway involves adenylate cyclase inhibition via $G\alpha_i$ coupling with subsequent decreases in intracellular cAMP [10]. The EP2 and EP4 receptors are expressed in almost all mouse tissues, while expression of EP1 is restricted to several organs, including the lung. EP2 expression is the least abundant of the EP receptors, however, several stimuli induce expression of EP2 [10].

2.2. Leukotrienes. LTs are also generated by liberation of AA from cell membranes (Figure 1). This is modified by a series of enzymes beginning with 5-lipoxygenase (5-LO), which acts in concert with 5-LO-activating protein (FLAP) to form leukotriene A_4 (LTA_4) [11]. LTA_4 can then be metabolized by LTA_4 hydrolase to form leukotriene B_4 (LTB_4). Alternatively, LTA_4 can be conjugated to reduced glutathione by leukotriene C_4 (LTC_4) synthase to form LTC_4. LTC_4 is exported from the cell by specific transporters [12] and can be acted on by extracellular peptidases to form LTD_4 or LTE_4. Leukotrienes C_4, D_4, or E_4 are collectively known as the cysteinyl leukotrienes (cysLTs).

Expression of 5-LO is tightly regulated and is primarily restricted to cells of the myeloid lineage, such as monocytes/macrophages, mast cells, eosinophils, and neutrophils. Although LT synthesis was once thought to be restricted to leukocytes, it has subsequently been shown that human bronchial epithelial cells and fibroblasts are capable of producing both cysLTs and LTB_4 [13, 14]. In addition, the intermediate LTA_4 can be transferred from an activated donor cell to a recipient cell. LTA_4 can then be metabolized to either LTB_4 or LTC_4 by LTA_4 hydrolase or LTC_4 synthase, respectively, in a process termed "transcellular biosynthesis" [15]. These enzymes are expressed in most tissues. In this way, other cell types, such as epithelial cells, can become an important source of LTs during an inflammatory response.

Like PGE_2, the effects of LTs are mediated by signaling through G protein-coupled receptors. Among the receptors for cysLTs, two have been thoroughly characterized. The cysLT1 receptor binds LTD_4 with high affinity and binds LTC_4 and LTE_4 with lower affinities [16]. The cysLT2 receptor binds LTC_4 and LTD_4 with equal affinity. A number of studies have alluded to the existence of additional cysLTR subtypes, although these have yet to be characterized [17]. The chemoattractant and proinflammatory effects of LTB_4 are mediated by the high-affinity B leukotriene receptor 1 (BLT1). A second receptor, B leukotriene receptor 2 (BLT2), binds LTB_4 with lower affinity, but its biological function remains poorly understood [18]. Studies in transfected cell lines have shown that the four LT receptors can couple to both $G\alpha_i$ and $G\alpha_q$ proteins to decrease cAMP and increase intracellular Ca^{2+}, respectively [19–23]. Studies in primary cells have yielded differing results and the specific signaling programs initiated by GPCRs remain to be dissected [24]. Within the human lung, cysLT1 mRNA is expressed in epithelial cells, bronchial smooth muscle cells, interstitial macrophages, and the nasal mucosa. CysLT2 is expressed by bronchial smooth muscle cells, interstitial macrophages, and nasal mucosa [17]. Human BLT1 is expressed primarily in leukocytes and its expression can be altered in response to various inflammatory stimuli [18, 25]. BLT2 is expressed

more ubiquitously, with high mRNA expression detected in the spleen and low levels in most human tissues, including the lung [26].

3. Eicosanoids and Immune Function

3.1. Prostaglandin E_2. PGE_2 regulates immune function in a myriad of ways that are likely to affect viral pathogenesis (Table 1). Widespread expression of COX-2 has been demonstrated in airway epithelial and resident inflammatory cells in the absence of overt inflammation, suggesting a role for COX-2 in regulation of human airway homeostasis [27]. High concentrations of COX products are present in the epithelial lining fluid of human airways, potentially playing a role in inhibiting lymphocyte activity and fibroblast proliferation in the absence of inflammation [28]. Additionally, constitutive secretion of PGE_2 by airway epithelial cells contributes to modulation of DCs under homeostatic conditions [29]. PGE_2 can promote inflammation through vasodilatory mechanisms, yielding edema, warmth, erythema, and passive leukocyte recruitment. However, PGE_2 is also capable of inhibiting neutrophil chemotaxis, phagocytosis, and bacterial killing [30, 31]. PGE_2 suppresses phagocytosis by nonalveolar monocytes/macrophages [32–35], and PGE_2 inhibits alveolar macrophage (AM) phagocytosis via a mechanism that involves EP2 activation and increases in cAMP [36]. Bacterial killing and reactive oxygen intermediate generation by AMs is also inhibited by PGE_2 in an EP2/EP4- and cAMP-dependent manner [37].

The production of various pro-inflammatory cytokines and chemokines is inhibited in the presence of PGE_2 [38, 39], while production of the anti-inflammatory cytokine interleukin (IL)-10 is enhanced [40]. PGE_2 suppresses production of the Th1 cytokines interferon (IFN)-γ and IL-12, leading to a Th2-polarized environment [41, 42]. However, a number of studies have also reported PGE_2-mediated enhancement of Th1 cytokine secretion and differentiation *in vivo* [43, 44]. The role of PGE_2 is not strictly suppressive, as it has been shown to promote certain pathways in immune differentiation. For example, PGE_2 can act on uncommitted B lymphocytes to promote isotype switching to IgE or IgG1 [45–47]. COX inhibitors inhibit antibody production in activated human B lymphocytes [48, 49]. PGE_2 augments IL-17 production and Th17 differentiation by increasing IL-23 production in T cells and dendritic cells [44, 50–53], an activity that likely occurs via EP2- and EP4-mediated increases in cAMP [54, 55]. Additionally, PGE_2 enhances the production of the proinflammatory cytokine IL-6 by leukocytes [56] and airway epithelial cells [57]. PGE_2 potently inhibits the production of a number of antimicrobial peptides (AMPs) such as human β-defensin by epithelial cells [58]. This effect of PGE_2 is likely to be relevant for viral pathogenesis, because AMPs can inhibit the replication of viruses [59, 60].

3.2. Leukotrienes. The diverse effects of LTs on innate immunity have been reviewed elsewhere [61] and are briefly summarized in Table 1. LTB_4 promotes neutrophil migration and survival [62, 63] and enhances neutrophil granule enzyme secretion [64] and superoxide anion generation [65, 66]. T lymphocyte recruitment to sites of inflammation can be induced by LTB_4 [67–70]. In addition to neutrophil and T cell trafficking, LTB_4 can promote the migration of dendritic cells (DCs) *in vitro* [71] and to draining lymph nodes as mice deficient in BLT1/2 show reduced DC migration [72]. Both cysLTs and LTB_4 can enhance Fcγ receptor-mediated phagocytosis by AMs, though by different mechanisms [24, 73, 74]. LTB_4 induces antimicrobial peptide release from neutrophils *in vivo,* in some cases inhibiting viral replication [75–77]. Lung generation of the proinflammatory cytokine TNF-α is enhanced by LTB_4 [78]. A number of studies have reported that LTB_4 acts synergistically with IL-4 to induce activation, proliferation, and differentiation of human B lymphocytes [79–81], although a separate study reported that 5-LO inhibitors actually enhanced B lymphocyte proliferation [82].

CysLTs can promote microvascular leak [11], enhance leukocyte survival [83, 84], and induce nitric oxide (NO) generation in neutrophils [66, 85]. CysLTs induce DC chemotaxis to CCL19 and DC trafficking to lymph nodes is impaired in LTC_4 transporter-deficient mice [12]. In addition, cysLTs have been suggested to play a role in allergen-induced DC migration from blood [86]. Addition of LTD_4 to activated B lymphocytes leads to a modest upregulation of IgE and IgG production [87]. CysLTs also play a role in regulation of a pulmonary Th2 response as mice deficient in LTC_4 synthase showed reduced Th2 cytokine mRNA expression and Ag-specific IgE and IgG1 in the lung [88]. CysLTs are recognized as important mediators in the pathogenesis of asthma by their ability to promote airway microvascular permeability, mucus secretion, and smooth muscle contraction [89–93].

The prostaglandins and leukotrienes modulate many host immune responses that are important contributors to viral pathogenesis, such as cytokine signaling, neutrophil and macrophage phagocytosis, trafficking and activation of DCs and T cells, and antibody production by B cells.

4. Eicosanoids and Respiratory Viruses

4.1. Influenza. Influenza infections account for over 200,000 hospitalizations annually in the USA [94]. In addition to hospitalizations, influenza is also associated with a substantial number of outpatient visits each year, causing considerable healthcare burden and economic costs. Influenza upregulates COX-2 expression both *in vitro* and *in vivo,* and it has been suggested that COX hyperinduction contributes to the exaggerated cytokine response observed in severe human H5N1 infections [95–97]. Alteration of the COX pathway has contrasting effects on inflammatory responses to influenza virus depending on the model of pharmacologic inhibition (COX-1- or COX-2-specific or dual inhibition) or of genetic deficiency. Treatment of influenza-infected mice with celecoxib, a selective COX-2 inhibitor, did not significantly affect viral titers or disease severity, although treatment did suppress production in the lung of the proinflammatory cytokines tumor necrosis factor- (TNF-) α, IL-6 and granulocyte-colony stimulating

Table 1: Effects of PGE_2 and leukotrienes on immune function.

	PGE_2	LTB_4	cysLTs
Neutrophils	Inhibits neutrophil chemotaxis, phagocytosis, and bacterial killing	Promotes neutrophil chemotaxis, ROS generation, and survival	Induces NO generation in neutrophils
Macrophages	Inhibits AM phagocytosis, ROS generation, and bacterial killing	Enhances AM phagocytosis	Enhance AM phagocytosis
T cells	Promotes Th17 differentiation	Induces T cell recruitment	Enhances Th2 response
B cells/Antibody Production	Promotes isotype switching to IgE and IgG1	Induces activation, differentiation, and proliferation of B cells	Upregulate IgE and IgG1 production by B cells
Dendritic Cells	Varies	Promotes DC migration	Promotes DC migration
Cytokines	Suppresses IFN-γ and IL-12 production Enhances IL-10 and IL-6 production	Enhances TNF-α production	Enhances IL-5, IL-13, and eotaxin expression
Antimicrobial Peptides	Inhibits AMP production by epithelial cells	Induces AMP production by neutrophils	Unknown

factor (G-CSF) [98]. In contrast, influenza infection of mice genetically deficient in COX-2 resulted in reduced mortality, inflammation, and cytokine responses compared to infection of wild-type control [99]. Peak lung viral titers were significantly elevated in COX-$2^{-/-}$ mice but returned to levels seen in wild-type mice by day 6, suggesting a role for COX-2 in controlling early viral replication but not in virus clearance. Interestingly, levels of PGE_2 in influenza-infected COX-$2^{-/-}$ mice were equivalent to levels measured in infected wild-type mice. The lack of PGE_2 deficiency in COX-$2^{-/-}$ mice could be due to compensatory upregulation of COX-1 activity, as has been described before [100].

Mice infected with highly virulent H5N1 and treated with a combination of celecoxib, the neuraminidase inhibitor zanamivir, and mesalazine (an aminosalicylate drug that exhibits weak 5-LO and COX inhibition [101]) showed significantly improved survival even when treatment was delayed 48 hours [102]. The beneficial effect of celecoxib and mesalazine likely stemmed from their effects on immunopathology, as mice treated with triple therapy had similar viral loads as those treated with zanamivir alone. Triple therapy significantly reduced levels of the proinflammatory cytokines IL-6, TNF-α, and IFN-γ.

Another group treated influenza-infected mice with paracetamol (acetominophen), a selective inhibitor of COX-2 [103, 104]. Paracetamol-treated mice had improved lung function, and reduced immunopathology compared to control mice. A separate group of mice treated with celecoxib also showed improvements in cellular infiltrates, lung function and pathology. However, the degree of improvement was generally less than that seen in paracetamol-treated mice. In contrast to mice genetically deficient in COX-2 [99], paracetamol- and celecoxib-treated mice had viral loads equivalent to those in untreated control mice. Virus-specific $CD4^+$ and $CD8^+$ T cell numbers were not altered in treated mice, and treatment with paracetamol or celecoxib did not interfere with the establishment of protective immunity to a second infection with a different influenza subtype.

The significantly increased viral titers seen in COX-$2^{-/-}$ mice but not observed in mice treated with COX-2 inhibitors could be due to a functional defect in innate immunity, as COX products are known to be involved in modulating the innate immune response [105]. In addition, COX-$2^{-/-}$ mice have a complete loss of COX-2 activity, whereas mice treated with inhibitors still retain some COX-2 activity due to insufficient inhibition by the drug. COX-$2^{-/-}$ mice had levels of PGE_2 in bronchoalveolar lavage (BAL) fluid similar to wild-type mice, suggesting that the effects of COX-2 deficiency in this model may not be due to lack of PGE_2. As COX-$2^{-/-}$ deficiency is likely to affect the production of other prostaglandins (such as TXA_2, PGD_2, PGF_2, and PGI_2), it is possible that decreased levels of one of the other COX products are responsible for increased survival.

Influenza infection upregulates 5-LO expression and/or levels of LTs in cell lines as well as in lungs of infected mice and humans [106–108]. However, few studies have examined influenza infection in the context of altered 5-LO production (either due to genetic deficiency or pharmacologic inhibition). One study has reported a beneficial effect of exogenous LTB_4 administration during influenza infection of mice [75]. Mice treated daily with LTB_4 had significantly reduced lung viral loads. The lungs of LTB_4-treated mice showed increased levels of multiple antimicrobial peptides, decreased inflammatory cell infiltration, and partially restored lung architecture. The antiviral effect of LTB_4 was mediated by neutrophils and the high-affinity BLT1 receptor, as viral loads were unaffected in neutrophil-depleted or BLT1-deficient mice. LTB_4 treatment of primary human neutrophils in this study induced antimicrobial peptide release and decreased influenza titers, demonstrating that the effects of LTB_4 on neutrophils are similar in both mice and humans. This is in agreement with another study, in which human

neutrophils treated with LTB_4 showed significantly more myeloperoxidase (MPO) activity and α-defensin production than untreated cells, and LTB_4-treated neutrophils had enhanced virucidal activity against influenza virus, human coronavirus, and RSV [109]. The role of cysLTs during influenza infection has yet to be defined in detail. Enhanced levels of cysLTs seen in COX-$2^{-/-}$ mice infected with influenza are associated with increased survival [99], but whether the decreased mortality in COX-2-deficient mice is directly due to cysLTs in this model is unknown.

The beneficial effects of COX-2 deficiency may also be due to shunting of released AA to the 5-LO pathway. A number of reports suggest that COX inhibitors enhance production of LTs [110, 111]. Indeed, COX-$2^{-/-}$ mice showed higher BAL fluid levels of cysLTs than wild-type mice following infection with influenza. However, in mice treated with a combination of zanamivir, celecoxib, and mesalazine, increased survival was associated with lower LT levels and higher PGE_2 levels in the treated mice compared to wild type. The discrepancies in COX and 5-LO products in these models may reflect the different pathophysiology of the influenza strains used. Perhaps increased LT production during severe H5N1 infection promotes inflammation and local tissue damage, while PGE_2 provides a balancing protective influence. In contrast, during infection with the less virulent H3N2 virus, enhanced LT production may contribute to virus clearance without a detrimental effect on host inflammation. However, in the case of either virus lower levels of the proinflammatory cytokines IL-6, TNF-α, and IFN-γ were correlated with decreased morbidity and increased survival. Other differences in the studies could be accounted for by differences in virus subtype, virus inoculum, mouse strain, or drug dose and delivery method. However, partial COX inhibition by pharmacologic intervention appears to be beneficial in reducing immunopathology while still controlling viral replication during influenza infection in mice.

4.2. Respiratory Syncytial Virus. Respiratory syncytial virus (RSV) is the leading cause of bronchiolitis and pneumonia in infants [112, 113]. RSV is also a significant pathogen in the elderly population, particularly those living in long-term care facilities or with underlying cardiopulmonary disease [114]. The immunocompromised are at risk for severe RSV infection, with mortality rates of up to 80% reported for RSV pneumonia [115]. RSV induces PGE_2 release *in vitro,* in animal models, and in lungs of infants with RSV bronchiolitis [116–119]. Treatment with COX inhibitors reduces RSV replication *in vitro* and diminishes immunopathology *in vivo.* Blocking PG production with NS-398, celecoxib, or the $cPLA_2$ inhibitor pyrrophenone reduced virus particle production in the A549 airway epithelial cell line [116]. COX inhibition also reduced transcription and production of the proinflammatory cytokines IL-8 and RANTES (CCL5). RSV-induced activation of interferon regulatory factor (IRF) and NF-κB activation were suppressed by a high concentration of celecoxib. Another study demonstrated that the nonselective COX inhibitor indomethacin decreased lung histopathology in RSV-infected cotton rats, but COX inhibition did not significantly affect viral replication [117].

RSV also induces production of LTB_4 and cysLTs in both animal models and infants afflicted with RSV bronchiolitis [119–125]. LT concentrations during RSV infection have been correlated with development of symptoms and in some reports are associated with disease severity [107, 122, 126, 127]. Treatment of RSV-infected mice with the 5-LO inhibitor zileuton reduced inflammatory cell numbers in the lung, prevented RSV-induced weight loss, and decreased RSV-induced airway constriction [122]. Viral titers were somewhat lower in the lungs of zileuton-treated mice, although the difference was not statistically significant. Even when administered after the emergence of respiratory symptoms, zileuton reduced airway resistance and weight loss compared to untreated mice. Treatment with the cysLTR1 antagonist MK-571 decreased RSV-induced airway hyperreactivity (AHR) [121]. In contrast to treatment with zileuton, MK-571 did not affect inflammatory cell recruitment or production of IL-4 and IFN-γ in RSV-infected mice. A possible effect of MK-571 on viral titers was not examined in this study.

Similar to highly virulent influenza H5N1, successful treatment of RSV infection may require the use of an antiviral agent in combination with an anti-inflammatory agent that limits immunopathology. In support of this, treatment of RSV-infected cotton rats with the RSV-specific humanized monoclonal antibody palivizumab and a glucocorticoid resulted in enhanced clearance of RSV and limited lung histopathology compared to controls [128]. Further support comes from a model of pneumonia virus of mice (PVM), a paramyxovirus that is a close phylogenetic relative of RSV. PVM infection increased levels of cysLTs in the lung [129]. In this model, administration of either the cysLT1 antagonist montelukast or the nucleoside analog ribavirin did not affect disease severity. However, combined therapy of montelukast with ribavirin substantially decreased morbidity and mortality of PVM-infected mice.

Administration of montelukast during primary RSV infection prevented enhanced AHR, airway eosinophil recruitment, and mucus overproduction upon reinfection [120]. Montelukast administered only during secondary infection did not affect this enhanced response. Previous studies have shown that LTs are only transiently elevated during the acute phase of infection and that levels drop to baseline shortly after [130]. This suggests that LT inhibitors may have a beneficial effect during the early phase of infection but may no longer be useful as treatment for the long-term airway dysfunction observed after RSV infection when LT levels are no longer elevated.

The above reports demonstrate a beneficial effect of 5-LO product inhibitors or cysLT1 receptor antagonists during primary infection with RSV. However, the studies in animal models used pharmacologic agents given to mice starting on the day before infection, whereas treatment in humans is typically initiated later during the course of infection after the emergence of symptoms. Delaying zileuton treatment until 3 days post infection, after respiratory symptoms emerged, still reduced clinical signs during primary RSV infection in

mice. However, there have been conflicting results when 5-LO inhibitors and cysLT antagonists were used as treatment in children with RSV bronchiolitis. One study suggested a beneficial effect of the cysLTR1 antagonist montelukast on lung symptoms after RSV bronchiolitis [131], but further studies have failed to corroborate these findings [132–134]. To our knowledge, there are no human studies that examine prophylactic administration of 5-LO pathway inhibitors or receptor antagonists to high-risk children. Further studies are needed to define the role of LT inhibitors in patients with primary RSV infection and in those experiencing persistent airway dysfunction after RSV.

While many viruses are capable of causing respiratory infections, relatively little is known about the contributions made by eicosanoids to the pathogenesis of respiratory viruses other than influenza and RSV. Rhinovirus (RV) infection increases expression of 5-LO, FLAP, and COX-2 in human bronchial cells [135]. In addition, cysLT levels in BAL fluid are increased upon rhinovirus infection in humans and correlate with emergence of upper respiratory symptoms [107, 135]. Adenovirus induces COX-2 expression and PGE_2 release in murine fibroblasts [136] and in human primary synovial fibroblasts [137]. Additional studies are necessary to examine adenovirus-induced PG production in lung-relevant cell types, but *in vivo* studies of human adenovirus pathogenesis are limited by the strict species specificity of adenoviruses. Using mouse adenovirus type 1 to study the pathogenesis of adenovirus respiratory infection [138] will provide a useful tool to define the roles of eicosanoids to adenovirus respiratory infection.

Human cytomegalovirus (HCMV) can also cause respiratory infections, although symptomatic disease is uncommon in immunocompetent individuals [139]. HCMV induces 5-LO expression and LTB_4 production [140] in vascular smooth muscle cells as well as COX-2 expression and PGE_2 production in fibroblasts [141]. COX-2 inhibition reduces levels of the immediate-early 2 mRNA and protein in addition to viral DNA replication and transcription of some early and late mRNAs. Treatment of HCMV-infected fibroblasts with COX inhibitors inhibits cell-to-cell spread of virus [142]. Of note, while many reports with other viruses have shown inhibition of viral replication or gene transcription by COX inhibitors at non-physiologic concentrations, these results with HCMV were obtained with concentrations of COX inhibitors that are achievable in human plasma. Although few studies have examined the effect of 5-LO products on HCMV pathogenesis, one study reported that exogenous LTB_4 inhibited reactivation of CMV following allogeneic bone marrow transplantation (BMT) in mice, demonstrating a beneficial effect for this LT [143].

5. Common Themes

From the data summarized above (see also Table 2), it is clear that the effect of COX or 5-LO inhibition or antagonism of cysLT receptors on host responses to respiratory viral infection is variable and in some cases may be pathogen- and/or model-specific. In general, COX inhibition or deficiency is associated with less exuberant inflammation and in some cases improved survival. COX products may play a role in controlling early viral replication, although this possible role is only evident for influenza infection in mice completely lacking COX-2 activity and not in mice treated with a COX-2 inhibitor. These data are consistent with the role of PGE_2 as an immunomodulatory mediator, balancing pro-inflammatory actions with suppressive effects on innate and adaptive immune function. Inhibition of LT production or signaling during respiratory viral infection is associated with less inflammation accompanied by variable (but generally beneficial) effects on lung physiology. However, administration of exogenous LTB_4 also blunted inflammatory responses to influenza virus in one study [75], suggesting that various 5-LO products may be differentially involved in promoting inflammation and affecting host immune responses to viral infection.

6. Therapeutic Implications

Respiratory viral infections cause substantial disease and are associated with significant morbidity, mortality, and healthcare utilization. Many antiviral drugs are available to treat infection with human immunodeficiency virus, and a smaller number of drugs such as acyclovir and ganciclovir are available to treat infections with herpesviruses such as herpes simplex virus, varicella zoster virus, and HCMV. In contrast, far fewer drugs are available to treat viruses that most frequently cause respiratory infections. Neuraminidase inhibitors such as oseltamivir and zanamavir can be used as prophylaxis to prevent infection by influenza virus or used to treat infection. Older drugs such as amantadine and rimantadine can also be used to prevent or treat influenza. However, the emergence of drug-resistant influenza strains has the potential to increasingly limit the utility of these drugs. The nucleoside analog cidofovir has been used to treat adenovirus infections, although it has substantial toxicities and no randomized clinical trials have been performed to show clinical benefit. Currently, there are no drugs that have consistently been shown to be safe and effective for the treatment of disease caused by infection with RSV, rhinovirus, human metapneumovirus, or other viruses that commonly cause respiratory infections.

Preventing virus-induced inflammation may serve as an important adjunct to any antiviral therapy. When antiviral drugs are not available, modulation of virus-induced inflammation by itself may serve as an effective strategy to treat disease caused by viruses. Drugs with the ability to modulate eicosanoid production, such as ibuprofen and acetaminophen, are already frequently used in patients with respiratory infections to alleviate fevers, myalgias, and nonspecific symptoms. Studies described above that show decreased virus-induced inflammation and increased survival in animals treated with an inhibitor of PG or LT synthesis or in PG- or LT-deficient animals support the potential benefit of this approach. Drugs that modulate eicosanoid production may be particularly useful to prevent or treat infections in patients with exaggerated eicosanoid production at baseline. For instance, exaggerated PGE_2 production in the setting of bone marrow transplantation

Table 2: Effects of PGE_2 and Leukotrienes on respiratory syncytial virus and influenza infection.

	PGE_2		Leukotrienes
	COX Inhibition	COX-2 Deficiency	
RSV	Reduction in viral replication *in vitro*		Reduction in pulmonary inflammatory, weight loss, and RSV-induced airway constriction in mice treated with 5-LO inhibitor
	Suppression of virus-induced cytokine production in vitro		
	No effect on viral replication in the lungs *in vivo*		CysLTR1 antagonism during primary infection prevents enhanced AHR upon reinfection
	Decreased lung pathology *in vivo*		Decreased RSV-induced AHR but no effect on cytokine production in mice treated with cysLTR1 antagonist
Influenza	No effect on viral replication or disease severity in micetreated with celecoxib	Decreased mortality, pulmonary inflammation and cytokine responses in COX-$2^{-/-}$ mice	Reduced lung viral loads and decreased pulmonary inflammatory in mice treated with exogenous LTB_4
	Suppression of virus-induced cytokine production in mice treated with celecoxib	Increased viral titers in lungs of COX-$2^{-/-}$ mice compared to controls	
	Improved survival and reduced proinflammatory cytokine levels in mice treated with zanamivir, celecoxib, and mesalazine		
	Improved lung function and reduced immunopathology in mice treated with paracetamol		

has been associated with increased susceptibility to bacterial infection that is linked to impaired neutrophil and macrophage phagocytosis and killing [144, 145]. Increased PGE_2 production has been reported in humans with a variety of disease states including cancer [146], aging [147], HIV infection [148], malnutrition [149, 150], and stem cell and solid organ transplant recipients [151, 152], making the potential benefits of this approach more widespread.

Any therapy that involves modulation of eicosanoid production must consider the potential for deleterious effects on the development of adaptive immunity and subsequent protection from secondary infection. PGE_2 plays an important role in optimal antibody synthesis, as COX inhibitors reduce antibody production in activated human B lymphocytes [48, 49]. In addition, mice genetically deficient in COX-2 produce significantly less IgM and IgG than wild-type mice [48]. There is evidence that COX-2 plays a role in potentiating antibody production in humans as well. Human volunteers challenged with RV showed increased nasal symptoms and a suppressed serum neutralizing antibody response when treated with aspirin or acetaminophen, suggesting a protective role for COX products in reducing symptoms and promoting an antibody response [153]. One large-scale study has been performed in which children were administered prophylactic paracetamol when receiving routine childhood vaccinations [154]. Antibody responses to several of the vaccine antigens were less robust in patients receiving prophylactic paracetamol. Evidence also exists that LTs, like PGE_2, promote appropriate antibody responses [79–81, 87], but the effect of 5-LO inhibitors and receptor antagonists on antibody production has not yet been described.

7. Conclusions

Eicosanoids modulate many host immune responses that are important contributors to viral pathogenesis. It will be essential to better define mechanisms underlying the effects of eicosanoids on both innate and adaptive immune responses to respiratory viral infection in order to develop therapies with maximal anti-inflammatory benefit and minimal impact on protective immune responses. For instance, the use of specific receptor agonists or antagonists may eventually provide a better-tailored approach than inhibitors of PG or LT synthesis to treat patients with respiratory viral infections. In general terms, however, alteration of eicosanoid production or antagonism of eicosanoid receptors has the potential to serve as a useful treatment strategy for respiratory viral infections.

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Review Article

Cyclooxygenase Inhibition in Sepsis: Is There Life after Death?

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Prostaglandins are important mediators and modulators of the inflammatory response to infection. The prostaglandins participate in the pathogenesis of hemodynamic collapse, organ failure, and overwhelming inflammation that characterize severe sepsis and shock. In light of this, cyclooxygenase (COX) inhibiting pharmacological agents have been extensively studied for their capacity to ameliorate the aberrant physiological and immune responses during severe sepsis. Animal models of sepsis, using the systemic administration of pathogen-associated molecular patterns (PAMPs) or live pathogens, have been used to examine the effectiveness of COX inhibition as a treatment for severe sepsis. These studies have largely shown beneficial effects on mortality. However, human studies have failed to show clinical utility of COX inhibitor treatment in severely septic patients. Why this approach "worked" in animals but not in humans might reflect differences in the controlled nature of animal investigations compared to human studies. This paper contrasts the impact of COX inhibitors on mortality in animal models of sepsis and human studies of sepsis and examines potential reasons for differences between these two settings.

1. Introduction

Sepsis is a major cause of morbidity and mortality worldwide [1], with more than 750,000 cases per year in the United States alone [2]. Despite improvements in diagnosis and therapeutics, there is an ongoing need for better treatments. Sepsis can be defined as a "systemic illness caused by microbial invasion of normally sterile parts of the body" [2], and it can be complicated by organ dysfunction (severe sepsis) or hypotension refractory to volume resuscitation (septic shock) [2].

Research into the fundamental mechanisms of sepsis has historically depended on animal models, with two primary approaches taken to model severe sepsis or septic shock. In one approach, live pathogens are used to cause sepsis. Examples of this approach include (1) inoculating the bloodstream or peritoneal cavity of animals with a single bacterial pathogen, (2) inducing peritonitis via cecal ligation and puncture (CLP), or (3) inoculating the peritoneal cavity of animals with fecal matter. The other approach induces the inflammatory response and complications of sepsis but is not *truly* sepsis since live pathogens are not utilized. In this approach, animals are exposed, usually via intravenous injection, to pathogen-associated molecular patterns (PAMPs) that trigger robust inflammatory responses by activating pathogen recognition receptor-based signaling cascades in the host. Typical PAMPs used to model sepsis include lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan, or mixed PAMPs delivered as inactivated (dead) bacteria.

Animal models of sepsis foster the *in vivo* investigation of signaling cascades that mediate this process. Over the past 50 years, lipid mediators known as prostaglandins (PGs) have garnered significant attention for their roles in mediating the inflammatory and immune response to severe infection. The PGs, oxygenated metabolites of arachidonic acid, are small molecules that have a myriad of roles in regulating pathophysiological responses during sepsis. The synthesis, catabolism, and signaling of PGs have been studied as targets in treating sepsis, particularly when used in combination with antimicrobial agents and supportive care. Early studies targeting PG synthesis in the treatment of sepsis involved

inhibiting the cyclooxygenase (COX) enzymes, the first committed enzymatic step in the metabolism of arachidonic acid into bioactive PGs [3, 4].

Despite encouraging results in animal models of sepsis and shock, human pharmacological trials of COX inhibitors have not provided consistent or significantly beneficial findings. The failure of COX inhibitors to significantly improve the outcome of humans with sepsis strongly dampened enthusiasm for targeting PG synthesis for this problem. Why this approach "worked" in animals but not in humans likely reflects important differences in the controlled nature of the investigations involving the former compared to the latter. This unstructured paper explores the impact of COX inhibitors on mortality in animal models of sepsis and human studies of sepsis and examines potential reasons for differences between these two settings. The possibilities for future use of PG-based strategies for treating sepsis are discussed.

2. Methods

Literature Review. Studies addressing the use of COX inhibitors and nonsteroidal anti-inflammatory drugs (NSAIDs) in animal and human studies of sepsis were identified using the PubMed database (National Library of Medicine, Bethesda, MD). The following search terms were used alone and in combination: "sepsis," "infection," "cyclooxygenase," "prostaglandins," "PGE_2," "receptors," and "hemodynamic". Additional references were identified within the bibliographies of PubMed-identified manuscripts. Searches were done for all available publication dates up until January 31, 2012.

Studies were included for analysis if COX inhibitors were administered to animals prior to or in response to the systemic (intravenous or intraperitoneal) administration of PAMPs or if COX inhibitors were administered to animals prior to or in response to a systemic infection model of sepsis. Such sepsis models with live organisms included both *monomicrobial* sepsis models, where a single species of bacteria was administered to animals systemically (intravenous or intraperitoneal) and *polymicrobial* sepsis models, where animals were infected with undefined mixtures of organisms either through the intraperitoneal introduction of stool or through CLP. Studies involving mice genetically deficient for COX isoforms were included as indicated. Human studies were included if COX inhibitors were administered to treat sepsis as defined by the authors of the studies.

The major outcome reviewed from these studies was the effect of COX inhibitors on mortality. Only studies that included data regarding mortality were included for such analyses.

3. Results

3.1. PAMP Models of Sepsis in Animals. A total of 43 manuscripts were identified that modeled sepsis in animals using the systemic administration of PAMPs to induce a physiological and immunological response similar to clinical sepsis [3–45]. There were 16 publications that did not report mortality data and were excluded from analysis [4–6, 8, 10, 13, 16, 17, 22, 26, 28–31, 36, 41]. Thus, 27 manuscripts were included that provided data regarding mortality after systemic PAMP exposure. Notably, two studies actually conducted studies on two types of species [23, 25], bringing the total number of studies evaluated to 29.

Mortality was assessed in seven different animal species across these studies (Figure 1(a)). Of these, 27 used LPS alone as the sepsis-inducing agent, while one study administered heat-killed *Corynebacterium parvum* prior to LPS [15] and one study used heat-killed Group B *Streptococcus* [34]. The source of the LPS was usually *Escherichia coli* but five studies used *Salmonella* LPS [4, 14, 25, 27, 45]. The PAMPs were usually delivered intravenously, although intraperitoneal approaches were also used. There was great heterogeneity among studies for the COX inhibitor used, the dose employed, whether the drug was administered before or after PAMP exposure, the route of administration of the drugs, and the number of doses of the COX inhibitor. A majority of studies used dual COX-1/COX-2 inhibitors but one study used isoform selective inhibitors [30]. One study was selected that did not use a COX inhibitor but a knockout of the COX-2 gene in mice [12].

The first animal study identified that examined the impact of COX inhibitors on PAMP-induced sepsis was a dog study published in 1962 by Northover and Subramanian [4]. This study was conducted before it was well established that antipyretic and analgesic agents function as COX inhibitors. Thus, the rationale for that work was that salicylate antipyretic agents (sodium salicylate and acetylsalicylic acid) might function as inhibitors of certain protease enzymes thought (at the time) to mediate host cardiovascular responses during bacterial sepsis [4]. While the authors examined actions of these agents on the hemodynamic effects of *Salmonella* LPS, they did not report on the effects of these medications on mortality. Such data were reported, however, in a 1967 study using acetylsalicylic acid in dogs exposed to *E. coli* LPS [3]. That study showed that acetylsalicylic acid significantly reduced the lethality of LPS in the dog model [3].

In total, the studies identified for this paper demonstrated that COX inhibition, and genetic COX-2 deletion [12], improved survival in 21 of 29 studies (72.4%) [3, 7, 9, 11, 12, 14, 18–21, 24, 27, 32, 34, 37, 39, 40, 42, 44, 45], caused no change in survival in 7 studies (24.3%) [15, 23, 33, 38, 43, 46, 47], and reduced survival in a single mouse study (3.4%) [35].

3.2. Infectious Animal Models of Sepsis. Seventeen studies were identified in 16 manuscripts that examined the impact of either pharmacological or genetic inhibition of COX enzymes and infection-induced sepsis [46–61]. There were three publications that did not report mortality data and were excluded from analysis [51, 52, 56]. Two studies included data from COX-2 null mice [53, 54].

The models of sepsis used in these studies included three mouse CLP studies [46, 53, 60], three rat studies involving

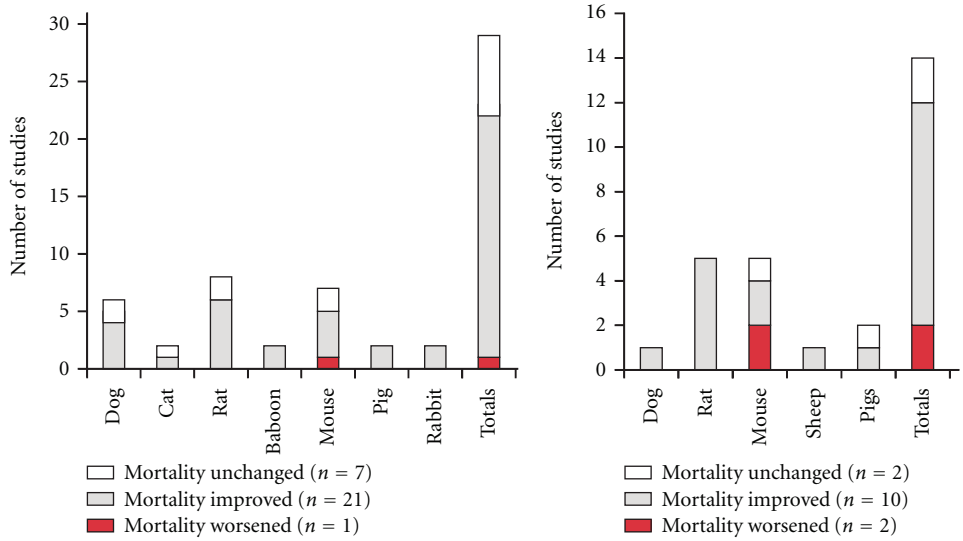

Figure 1: Influence of COX inhibitors on animal models of sepsis. (a) Studies were reviewed for experiments in which mortality was assessed for animals treated with COX inhibitors either before or after the systemic administration of pathogen-associated molecular patterns (generally, lipopolysaccharide). One mouse study (that showed a benefit to survival) included COX-2 knockout animals and not a pharmacological inhibitor. (b) Studies were reviewed for experiments in which mortality was assessed for animals treated with COX inhibitors either before or after the induction of systemic infection (see text for details). One mouse study (that showed a reduction in survival) included COX-2 knockout animals and not a pharmacological inhibitor.

the introduction of feces into the peritoneal cavity [49, 57, 61], two rat studies using systemic infection with live *E. coli* [48, 57], one rat study with systemic infection with live Group B *Streptococcus* [58], two canine studies with systemic infection with live *E. coli* [50, 51], three porcine studies with systemic infection with live Group B *Streptococcus* [47, 56, 59], one mouse study with systemic infection with live *Vibrio vulnificus* [55], one mouse study using live Group A *Streptococcus* [54], and one sheep study of infection with live *E. coli* [48].

Of the 14 studies evaluable, mortality was improved in 10 (71.4%), as depicted in Figure 1(b) [48–50, 54, 57–61].This included one of the two COX-2 knockout mouse studies [54]. Two studies showed no effect on mortality of COX inhibition [46, 47] and two mouse studies revealed increased death in either COX-inhibitor-treated mice [55] or a COX-2 knockout mouse model [53].

3.3. Human Studies of Sepsis. Three studies have examined the impact of COX inhibitor therapy on mortality in humans suffering from clinically defined sepsis [62–64], and one study [65] was a subgroup analysis of a larger study [62]. None of the three primary studies showed any positive or negative impact of COX inhibitors on mortality. Two used ibuprofen [62, 63] and one used lornoxicam, a drug relatively more potent against COX-2 than COX-1 [64]. The largest study was conducted by Bernard et al. and randomized 455 subjects to receive ibuprofen 10 mg per kilogram (maximal dose, 800 mg) over a period of 30 to 60 minutes every 6 hours for eight doses or placebo [62]. A similar but much smaller study was conducted by Haupt et al. and randomized 29 patients with sepsis to ibuprofen (600 mg or 800 mg intravenously followed by 800 mg per rectum every six hrs) or placebo [63]. The lornoxicam study by Memiş et al. included 40 subjects with sepsis randomized to receive either lornoxicam (8 mg administered intravenously every 12 hrs for six doses) or placebo [64].

The first human study examining the role of COX inhibitors in sepsis was conducted by Haupt et al. in 1991 [63]. This randomized, double-blind, multicenter study included 29 patients with clinical evidence of severe sepsis (16 were given ibuprofen and 13 were administered placebo). Eight of the ibuprofen-treated patients presented with shock and seven had the acute respiratory distress syndrome (ARDS), while four of the placebo-treated subjects had shock and four had ARDS. Nine patients in the COX inhibitor group died (56%) versus four in the placebo group (31%) (nonsignificant difference) [63].

In 1997, Bernard et al. conducted a larger, multicenter study that also compared ibuprofen with placebo in a randomized, blinded fashion [62]. There were 224 patients in the ibuprofen group and 231 in the placebo treatment arm. Most patients in both groups had two or three organ systems failing at study entry and nearly 50% of subjects had pneumonia in both groups [62]. Shock was present in 65% and 63% of patients in the COX inhibitor group and placebo group, respectively. Thirty-day mortality did not differ significantly in the drug-treatment and placebo groups (37% versus 40%) [62]. Notably, and perhaps relevant to the

findings of this study, acetaminophen use was permitted in both arms, and this agent was applied to 44% of subjects in the placebo group (but only 22% in the ibuprofen group) [62].

A substudy of this clinical trial was later published by Arons et al. [65], and it examined hypothermic patients with sepsis who were treated in a randomized, controlled study of ibuprofen at a dose of 10 mg/kg (maximum 800 mg) administered intravenously every 6 hr for eight doses compared with placebo. There were 44 subjects in that study, of which 13 received ibuprofen [65]. A significant reduction in 30-day mortality rate from 90% (18/20 placebo-treated patients) to 54% (13/24 ibuprofen-treated patients) was observed [65].

In 2004, a randomized, placebo-controlled study of lornoxicam was conducted by Memis et al. [64]. Of 40 subjects enrolled, half received the COX inhibitor and half received placebo. Shock was seen on admission in seven patients in the lornoxicam group and eight in the placebo group. The age range of subjects was wide and similar in both groups (for the 40 patients, 19–89 years old). Mortality was 35% in the lornoxicam group and 40% in the placebo group (nonsignificant difference) [64].

4. Discussion

Herein we review the impact of COX inhibition on mortality in animal and human models of sepsis. A major finding was that outcomes were better in animal studies than in human studies. While the reasons for this are unclear, it may be important for advancing new treatments for sepsis to more closely explore possible explanations.

When sepsis was modeled in noninfectious, PAMP-driven animal experiments, mortality was improved by COX inhibitors, in 72.4% of studies (Figure 1(a)). These studies spanned seven animal species and only a single mouse study revealed greater lethality when COX inhibitors were used. It is possible that heterogeneity in results related to differences in the dose and microbial source of PAMPs or the type, dose, and route of administration of the COX inhibitors used among studies. On balance, these data suggest that in addition to any effects on host defense mechanisms against live pathogens, COX metabolites increase the mortality resulting from an overwhelming host inflammatory response, possibly due to their importance in systemic vasodilation and renal blood flow [66]. Indeed, several of these studies showed improved hemodynamic parameters in animals treated with a COX inhibitor [3, 5–7, 10, 13, 17, 19, 21–24, 26, 28, 29, 31, 32, 40, 41, 43].

It is also notable that animals with actual infections causing sepsis fared better when COX inhibitors were used (Figure 1(b)). These results could be due to similar mechanisms that protected animals from PAMP exposure. However, in the setting of live infection, another determinant of outcome is the capacity of the host's immune system to eliminate invading pathogens. It is possible that COX inhibitors could influence host immune defense mechanisms since PGs are well known to regulate both innate and adaptive immunity [67–70].

Clearly, animal studies of infection-related sepsis and PAMP-associated inflammation have been able to demonstrate a benefit of COX inhibitor use. Why the lack of effect in human studies? For one, important differences exist in the experimental design between animal studies of sepsis and human clinical trials. It is likely that these differences confound potentially beneficial actions of COX inhibitors in human infection. Animal studies benefit from uniformity among the treated and control groups. Strong similarities (or identicalness) exist in parameters that might impact outcome, including age, gender, genetic background (in mouse studies particularly), general health, rearing environment, commensal microbiota. In addition, the insult (whether infectious or not) is generally highly defined and uniform in animal studies (e.g., all animals will receive the same dose of LPS or the same-sized cecal puncture wound). Animals have also not commonly been treated with antibiotics or other disease-modifying agents during studies of sepsis, which might otherwise alter the results. Lastly, in many animal studies of sepsis, COX inhibitors were given *prior* to the onset of overwhelming inflammation or infection, though studies giving the medication *after* the onset of sepsis have generally concurred [48, 57, 59, 71].

In stark contrast to the highly controlled animal studies, human clinical studies suffer from variability in almost every measurable aspect. Subjects with sepsis are not uniform in age, gender, comorbidities (or the presence of immunosuppression), the cause of sepsis (a major difference), the timing of therapy relative to the onset of symptoms, and so forth. Another major difference is that human subjects receive supportive care beyond that generally administered to animals, including intravenous fluids, vasopressive agents, mechanical ventilation, blood products, surgery, and (perhaps most importantly) antibiotics. Thus, the lack of impact of COX inhibitors in human studies may be due to a lack of similarity within and between cases and controls. It is also possible that the incremental effect (whether beneficial or detrimental) of COX inhibitors is too small to measure reliably when other supportive therapies are making more significant impacts on patient recovery.

The lack of clear benefit for COX inhibitors in human studies of sepsis begs the question of whether there is any worth in continuing to investigate PG synthesis and signaling cascades as targets for sepsis treatment. Given the risk of "throwing out the baby with the bathwater," it is important to determine whether the wealth of animal studies has provided a clue to novel therapies that remains undiscovered. For example, COX inhibitors might be most beneficial if given early in sepsis when patients are otherwise relatively robust: young, free of comorbidities, and so forth. Unfortunately such a patient population is a minority in the world of sepsis.

An alternative notion to explain the lack of benefit of COX inhibitors in human sepsis is that specific prostanoid molecules might need to be targeted, as opposed to blocking the most proximal committed step in PG synthesis. Perhaps in human cases of sepsis some PGs are helpful and others maladaptive. This approach has been taken in both animal and human studies [72]. For example, inhibiting the synthesis of thromboxane [73, 74] has been attempted

in small human studies of sepsis and acute respiratory distress syndrome with disappointing results. Animal models have been used more extensively to study select COX-derived eicosanoids [28, 75]. An example is provided by the molecule PGE_2, whose synthesis and signaling is increasingly being investigated as a target for immunotherapy in severe infections [54, 76–78].

In summary, myriad highly controlled animal models of sepsis provide a strong rationale for the targeting of PGs in the treatment of sepsis. However, the relatively small number of human studies has failed to support this approach. While the reasons for these differences are unclear, future studies are warranted to identify either particular human populations who might benefit from COX inhibitor treatment during sepsis or to identify particular prostanoids whose synthesis or signaling pathways can be specifically targeted during sepsis management.

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Review Article

Lipid Bodies: Inflammatory Organelles Implicated in Host-*Trypanosoma cruzi* Interplay during Innate Immune Responses

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The flagellated protozoa *Trypanosoma cruzi* is the causal agent of Chagas' disease, a significant public health issue and still a major cause of morbidity and mortality in Latin America. Acute Chagas' disease elicits a strong inflammatory response. In order to control the parasite multiplication, cells of the monocytic lineage are highly mobilized. Monocyte differentiation leads to the formation of phagocytosing macrophages, which are strongly activated and direct host defense. A distinguishing feature of Chagas' disease-triggered macrophages is the presence of increased numbers of distinct cytoplasmic organelles termed lipid bodies or lipid droplets. These organelles are actively formed in response to the parasite and are sites for synthesis and storage of inflammatory mediators. This review covers current knowledge on lipid bodies elicited by the acute Chagas' disease within inflammatory macrophages and discusses the role of these organelles in inflammation. The increased knowledge of lipid bodies in pathogenic mechanisms of infections may not only contribute to the understanding of pathogen-host interactions but may also identify new targets for intervention.

1. Introduction

The flagellated protozoa *Trypanosoma cruzi* is the causal agent of Chagas' disease (aka American trypanosomiasis) discovered at the beginning of the twentieth century by the Brazilian physician Carlos Chagas [1]. This disease remains a major problem with a great impact on public health in the Latin America. Chagas' disease affects nearly 8 million people and 28 million people are at risk of acquiring the disease in 15 endemic countries of Latin America [2]. Unfortunately, there is no vaccine available to prevent Chagas' disease [3].

T. cruzi is transmitted to humans primary through the feces of triatomine insects, at bite sites or in mucosa, through blood transfusion or orally through contaminated food. The parasite then invades the bloodstream and lymphatic system, and becomes established in the muscle and cardiac tissue, digestive system, and phagocytic cells [4]. *T. cruzi* may also be transmitted from mother to child across the placenta and through the birth canal, thus causing abortion, prematurity, and organic lesions in the fetus [4].

Chagas' disease is characterized by an acute phase with or without symptoms, and with entry point signs (inoculation chagoma or Romaña's sign), fever, adenomegaly, hepatosplenomegaly, evident parasitemia, and an indeterminate chronic phase (asymptomatic, with normal results from electrocardiogram and X-ray of the heart, esophagus, and colon) or with a cardiac, digestive, or cardiac-digestive form [4]. The factors that determine the distinct clinical outcomes, leading to a mild or to a severe form of the disease, are not completely understood. In fact, the most intriguing challenge

to understanding the pathophysiology of Chagas disease still lies in the complex host-parasite interrelationship (reviewed in [5]).

The parasite has an obligate intracellular, proliferative, nonflagellate form, called amastigote. After many division cycles, the amastigote forms convert into a flagellate form, the infective trypomastigote. Due to the high number of parasites into the host cell cytoplasm, the cell membrane disrupts and the infection spreads, affecting different organs (reviewed in [4, 6]).

Acute Chagas' disease elicits a strong inflammatory response. In order to control the parasite multiplication, cells of the monocytic lineage are highly mobilized (Figure 1). There is an intense migration and extravasation of monocytes from the bloodstream into target organs, mainly the heart (Figures 1 and 2). Monocyte differentiation leads to the formation of phagocytosing macrophages, strongly activated and involved in inhibiting parasite replication in the myocardium and other tissues [7, 8] (Figure 1). The ability of activated macrophages to process and present antigens, produce cytokines, and provide costimulatory signals demonstrates their pivotal role in initiating immune responses. The importance of these cells to the host defense has been pointed out by us and other groups during the *in vivo* acute *T. cruzi* infection in both humans and experimental models [7–10].

Because macrophages are key players in the initial resistance to the *T. cruzi* infection, a better understanding of their responses to the parasite is hence crucial for the development of appropriate therapeutic interventions and Chagas' disease control. A distinguishing feature of Chagas' disease-triggered macrophages is the presence of increased numbers of distinct cytoplasmic organelles termed lipid bodies (aka lipid droplets) [11]. Lipid bodies are lipid-rich organelles found in small numbers in most eukaryotic cells as roughly spherical organelles, comprised of an outer monolayer of phospholipids, a core containing neutral lipids, and variable protein composition. In contrast to other organelles, lipid bodies lack, therefore, a delimiting unit membrane structure (reviewed in [12]). Analysis of the fatty acid composition of the phospholipids revealed that they are structurally distinct from the phospholipids of the rough endoplasmic reticulum (ER) and from cholesterol/sphingolipid-rich microdomains. Unique features of lipid bodies include the abundance of unsaturated fatty acids in lyso-phosphatidylcholine and the relative abundance of phosphatidylcholine with 2 monounsaturated acyl chains [13]. The hydrophobic core of lipid bodies is occupied by triacylglycerols, diacylglycerols, retinyl esters, free cholesterol, and cholesterol esters in various ratios depending on the cell type [14–16].

Leukocyte lipid bodies contain several functionally diverse types of proteins, including structural proteins, metabolic enzymes, and kinases. Lipid body-specific structural proteins, the PAT family of proteins—Perilipin, adipose-differentiation-related protein (ADRP) [17] and tail-interacting protein of 47 kDa (TIP47) [18]—are found at the circumferential rim of lipid bodies. Moreover, a number of small GTPases of the Rab family, considered critical regulators of vesicular traffic and organelle interaction, and a variety of other proteins are described in lipid bodies [17, 19, 20].

In the past, lipid bodies were largely associated with lipid storage, but it is now recognized that lipid bodies are dynamic and functionally active organelles linked to diverse biological functions, such as lipid metabolism, cell signaling, and membrane trafficking (reviewed in [12, 21]). Lipid body has also been associated to immunoregulatory function in a number of human inflammatory diseases including inflammatory arthritis [22], acute respiratory distress syndrome [23], hypereosinophilia syndrome [24], and mycobacterial infection [25, 26], in addition to be related to neoplasic and emerging metabolic diseases, such as atherosclerosis, diabetes, and obesity (reviewed in [27, 28]).

Since the first report on lipid body formation in response to the *in vivo* acute *T. cruzi* infection by our group in 2003 [11], we have been investigating these organelles as key players in the host-parasite interaction and markers of macrophage activation during infectious diseases [6, 29–31]. This review covers current knowledge on lipid bodies triggered by the acute Chagas' disease within inflammatory macrophages and discusses the role of these organelles in inflammation. The increased knowledge of lipid bodies in pathogenic mechanisms of infections may not only contribute to the understanding of pathogen-host interactions but may also identify new targets for intervention [12, 29, 31].

2. *Trypanosoma cruzi* Induction of Lipid Body Formation

Although accumulation of lipid bodies has been documented for nearly 30 years in leukocytes and other cells in different inflammatory diseases [22–24, 32], the observation of lipid body formation in response to an *in vivo* infectious disease dates to 2003 [11]. By investigating inflammatory macrophages from rats infected with a virulent strain of *T. cruzi* (Y strain), it was observed a significant increase of the lipid body numbers in peritoneal macrophages at day 6 and 12 of the infection. While control peritoneal macrophages presented $\sim 2.19 \pm 0.4$ (mean ± SEM) lipid bodies/macrophage, peritoneal macrophages from infected animals showed $\sim 18.09 \pm 1.4$ at day 12 of infection [11].

In vitro, lipid body accumulation has been observed within peritoneal macrophages isolated from mice and cultured with *T. cruzi* for 24 h [35] (Figures 3(a)–3(d)). At this time of infection, both the cells containing internalized parasites as well nonparasitized cells show increased number of lipid bodies compared to control, noninfected cells, suggesting a bystander amplification of the response (Figure 3(b)). Interestingly, parasitized cells show significant higher number of lipid bodies (threefold) compared to nonparasitized cells, indicating that the uptake of the parasite directly induces formation of lipid bodies [35] (Figure 3(b)). D'Avila and colleagues have also demonstrated that lipid body formation in macrophages in response to the *T. cruzi* infection occurs through a Toll-like receptor-2- (TLR2-)

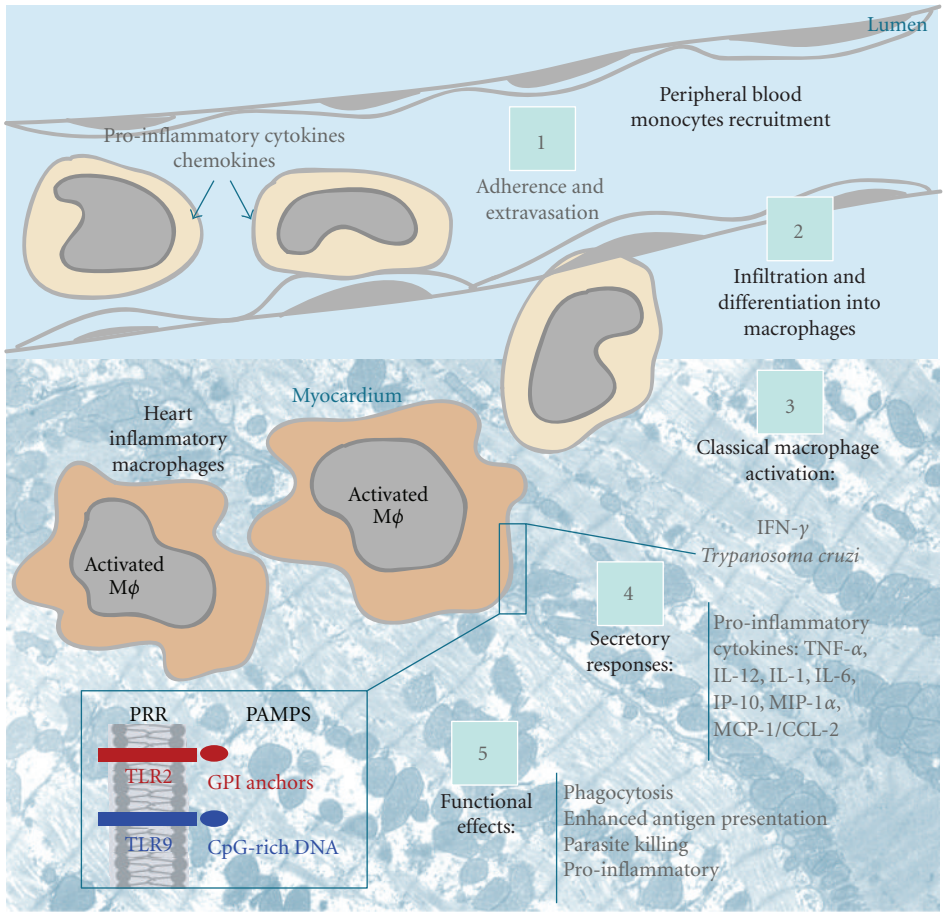

Figure 1: Activation of heart inflammatory macrophages during the acute infection with *Trypanosoma cruzi*. In response to acute infections, a cascade of events recruits cells derived from monocytic lineage from the peripheral blood into heart. This cascade culminates in a strong activation of macrophages. Classical activation of macrophages involves the key cytokine interferon gamma (IFN-γ) and *T. cruzi* components (GPI anchors and CpG-rich DNA). These parasite products are recognized at the macrophage surface by Toll-like receptors (TLRs)-2 and 9, respectively. These receptors are a class of pattern recognition receptors (PRRs), which initiate an immune response and directly activates macrophages. Additionally, TLR-2 can heterodimerizate either TLR1 or TLR6 to recognize *T. cruzi* GPI anchors [33]. TNF-α is one of the most efficient activators of macrophages to a trypanocidal function. PAMPS, pathogen-associated molecular patterns; GPI, glycosyl phosphatidy linositol; TNF-α, tumor necrosis factor-alpha; MIP-1α, macrophage inflammatory protein α; MCP-1/CCL-2, monocyte chemoattractant protein-1; IP-10, inducible protein 10.

dependent mechanism, demonstrating a mechanism involving surface receptors in this event [35].

As noted, the accumulation of newly recruited macrophages in the heart is one of the main findings of the acute *T. cruzi* infection and it is correlated with the intense myocardium parasitism which occurs during the early infection in both experimental models and humans (reviewed in [6]) (Figure 2). At day 12 of *T. cruzi* infection in rats, is observed the most intense inflammatory process and parasitism in the heart compared to other points during the acute phase [7]. Ultrastructural analysis of this organ showed numerous infiltrating macrophages with lipid bodies prominently increased in number and size [11] (Figure 4(a)). Inflammatory heart macrophages, evaluated by quantitative electron microscopy, exhibited a mean of 8.3 lipid bodies/cell section (range of 1–25) at the same time of infection whereas control noninflammatory macrophages showed a mean of 2.6 lipid bodies/cell section (range of 0–3) [11].

One intriguing aspect of lipid bodies is their osmiophilia, which is dependent on the cell type and can change during elicited inflammatory responses (reviewed in [12]). In inflammatory macrophages, the lipid body density can consistently change during pathogen infections, as revealed by ultrastructural studies [29, 31]. Based on osmiophilia, lipid bodies were identified and quantitated as light-dense, electron-dense, and strongly electron-dense organelles within inflammatory macrophages from different origins, mainly from the heart [29]. *T. cruzi* infection induces a significant increase in the numbers of light-dense lipid bodies compared to noninfected controls which show lipid bodies preferentially as electron- dense organelles (Figure 4(a)). Of note, lipid bodies change consistently their osmiophilia in macrophages stimulated *in vivo* with higher parasite load in irradiated-infected rats. These animals were exposed to a single, high dose of gamma irradiation 1 day before infection, which depletes the humoral and cellular

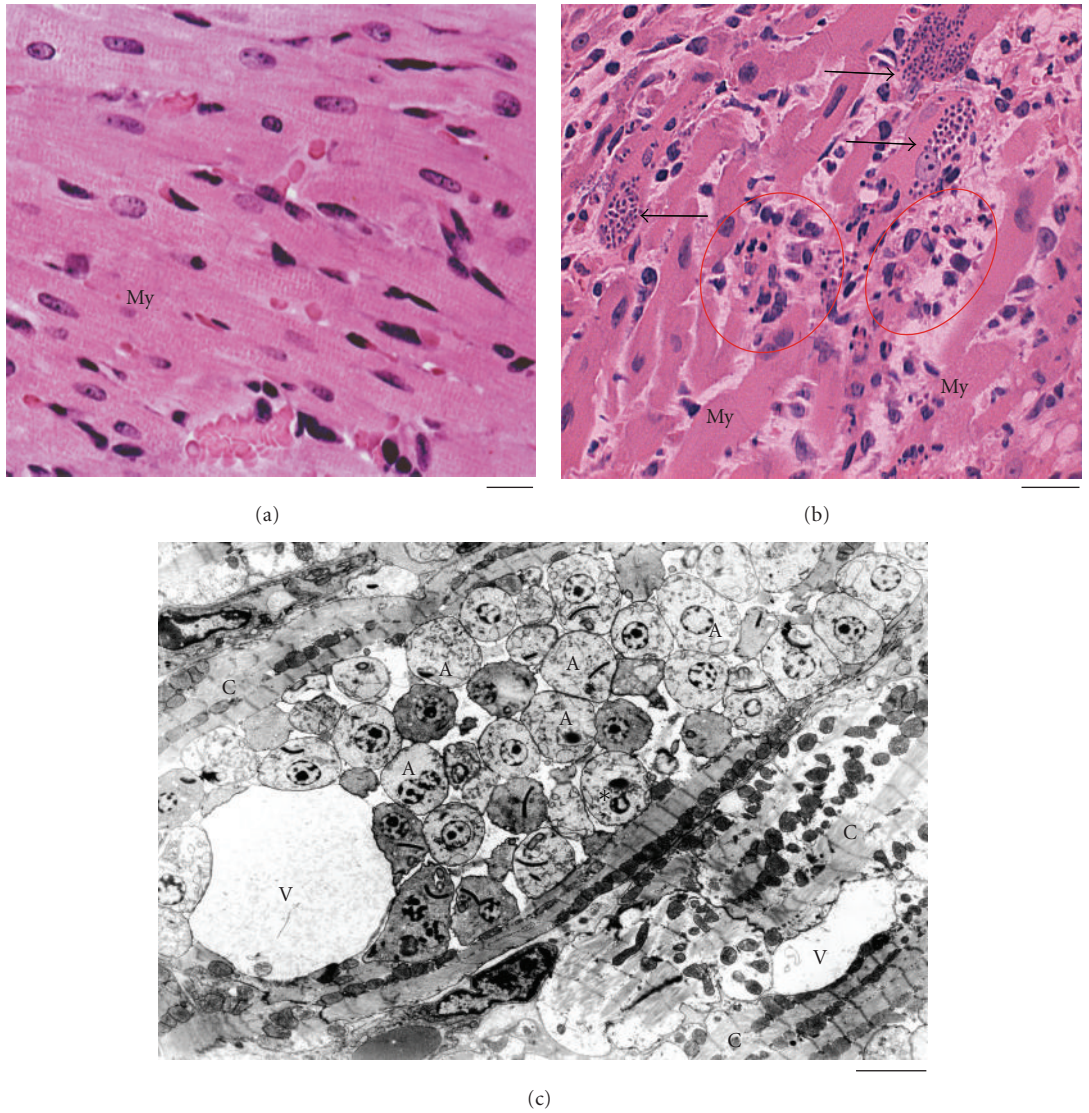

Figure 2: Morphological aspects of the heart from noninfected and infected rats at 12 days of infection with *Trypanosoma cruzi*. (a) Histological view of the myocardium from an uninfected rat. In (b), nests of amastigotes (arrows), the intracellular form of the parasite, and inflammatory processes (circles) characterized by predominance of mononuclear cells are observed in the myocardium (My). Semi-serial 5 μm-thick sections of the heart were cut, stained by haematoxylin and eosin and examined for qualitative evaluation of the inflammatory and degenerative processes and quantification of parasitism [8, 34]. In (c), an electron micrograph of a parasitized cardiomyocyte (c) showing vacuoles (V) and many amastigotes (A). Data are representative of three independent experiments. Four to six rats per group. Panel (a) was reprinted from [9] with permission. Scale bar, 15 μm (a, b); 1 μm (c).

immune responses except for the phagocytic activity of macrophages. Inflammatory macrophages from irradiated-infected animals show an increase in the numbers of both light-dense and strongly electron-dense lipid bodies compared to infection alone [29] (Figures 4(b) and 4(c)). Lipid body morphological changes, including alterations in osmiophilia, may reflect differences in lipid composition, stages of formation of new lipid bodies, mobilization, and/or neutral lipids/phospholipids ratio within lipid bodies. In addition, these morphological changes highlight lipid bodies as dynamic organelles, able to consistently change their structure in concert with cell activation [29]. In fact, lipid bodies in activated macrophages can be imaged as heterogeneous organelles, with lucent areas, granular, and/or membranous internal structures [29]. Of interest, by proteomic and ultrastructural studies, we have defined lipid bodies as organelles with internal endoplasmic reticulum-(ER-) like membranes and ER luminal proteins, suggesting a model by which enveloped ER-membranes and domains form lipid bodies [19].

Another morphological feature of lipid bodies is their considerable size variation. For example, in scoring the diameters of lipid bodies within macrophages from rats experimentally infected with *T. cruzi*, 74% of lipid bodies

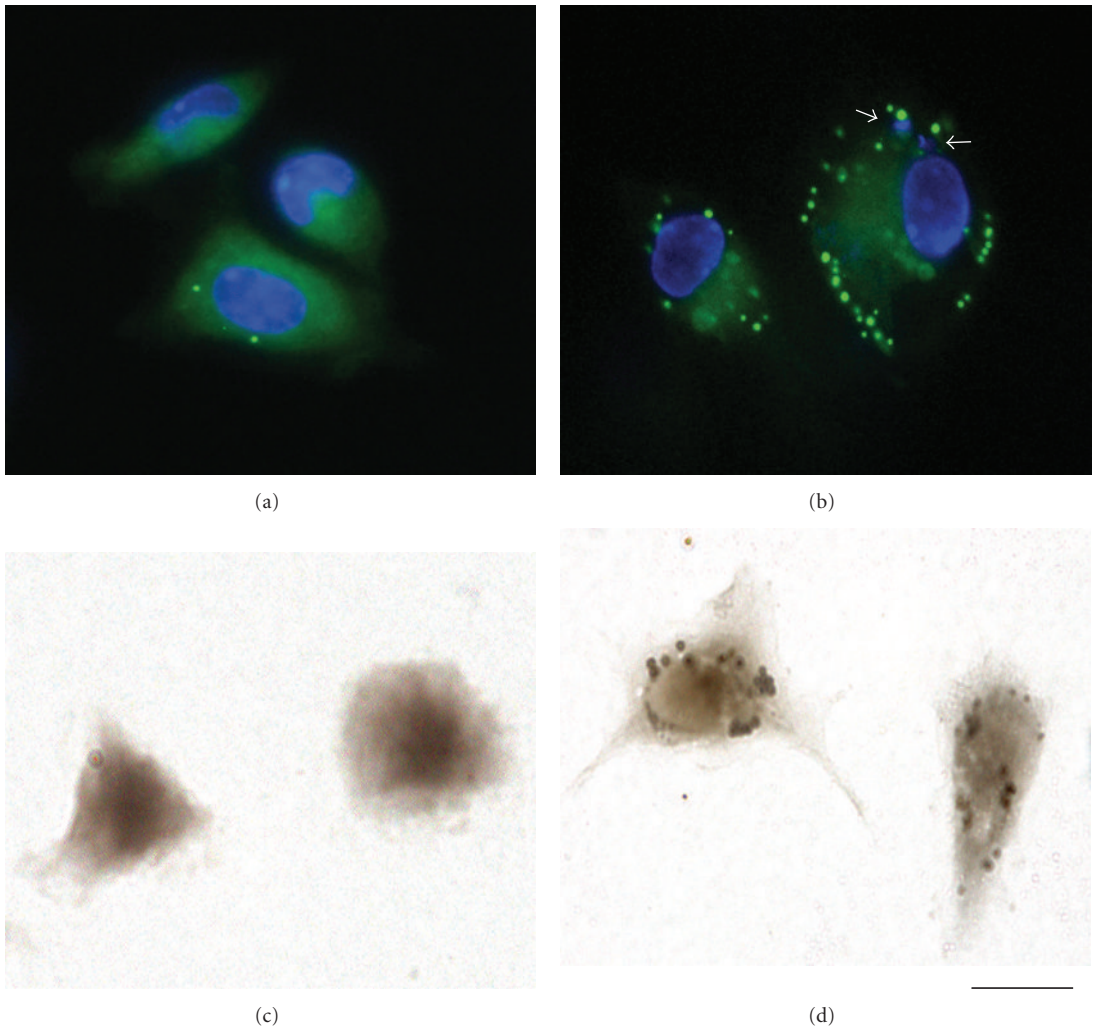

Figure 3: *Trypanosoma cruzi* infection induces macrophage lipid body formation. Peritoneal macrophages isolated from mice were cultured or not with *T. cruzi* and the formation of lipid bodies analyzed after 24 h by staining with BODIPY 493/503 (a and b), a fluorescent lipid probe for highly hydrophobic environments [35] or osmium tetroxide (c and d). While uninfected cells (a, c) have small number of lipid bodies, infected cells (b and d) show increased number of these organelles. Lipid bodies are seen as green (a and b) or brownish (c and d), round organelles. Nuclei of macrophages and internalized parasites (arrows) were stained with DAPI (4′,6-diamidino-2-phenylindole; blue). Scale bar, 10 μm.

had size <0.5 μm in noninfected whereas 54% of lipid bodies from infected animals were >0.5 μm, reaching up to 3 μm (Figure 4(c)). Increase of the parasite burden induced by gamma irradiation triggered significant formation of large lipid bodies within inflammatory macrophages, with diameters around 4 μm [29]. These findings reveal that not only the number but also the osmiophilia and size of lipid bodies represent structural indicatives of the participation of these organelles in innate immune responses [29].

Accumulation of lipid bodies within macrophages has also been documented during other infectious diseases, for example, during the progression of tuberculosis caused by *Mycobacterium tuberculosis* in both humans and experimental settings [25, 36] and in the course of leprosy, caused by *Mycobacterium leprae* [37]. In experimental studies with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), it was found that this pathogen is capable of inducing a dose- and time-dependent increase on LB formation within pleural and peritoneal macrophages [31, 38].

In addition to inducing lipid body formation [11], the infection with *T. cruzi* elicits a close interaction of lipid bodies with phagosomes within macrophages and even an apparent translocation of lipid bodies into these vacuoles, suggesting a discharge of the lipid body content [29]. However, the meaning of this interaction is still unknown. The lipid body-phagosome association as well as the internalization of lipid bodies by phagosomes during the acute *T. cruzi* infection may represent different stages of phagosome maturation leading to killing of the parasite or favor the parasite survival [29].

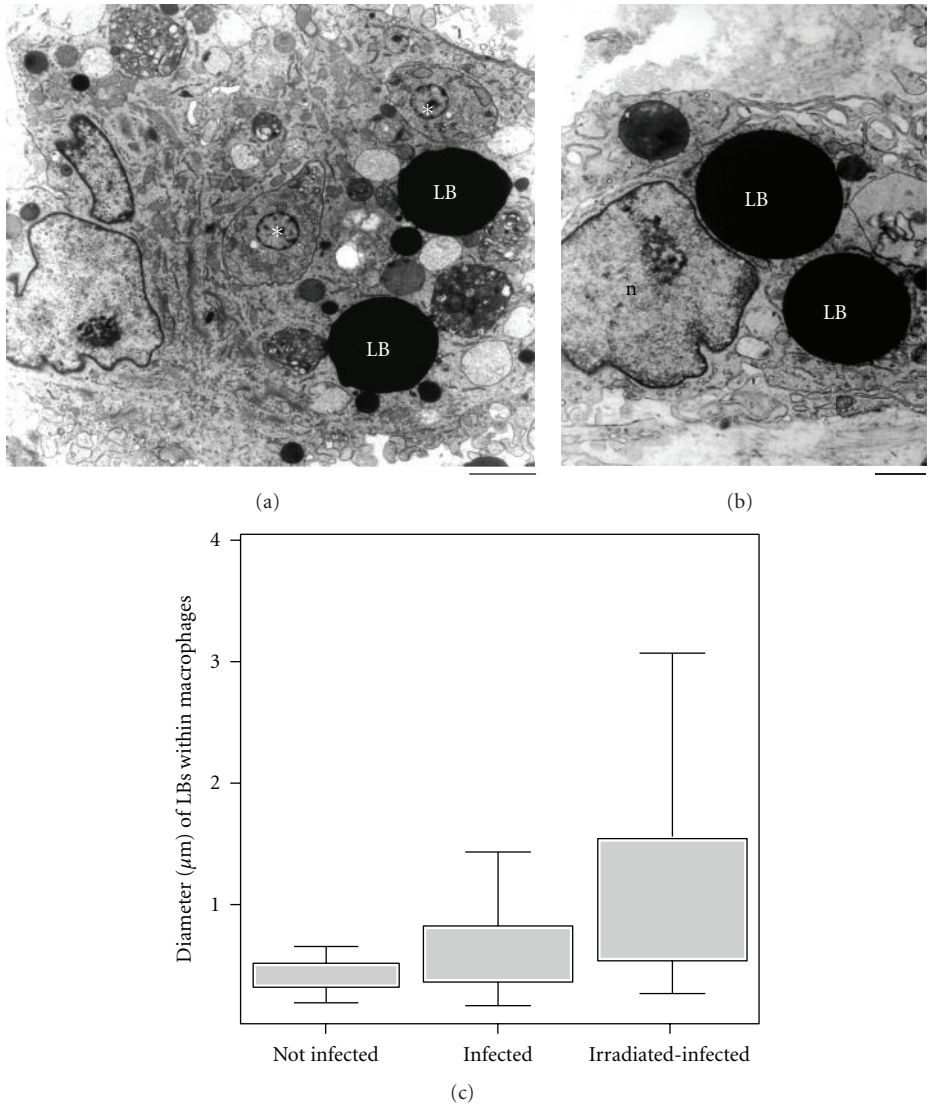

Figure 4: Lipid bodies (LB) within heart inflammatory macrophages increase in size in response to acute *Trypanosoma cruzi* infection and parasite load. (a) Strongly electron dense LBs from an infected animal are observed in the cytoplasm n conjunction with free amastigote forms of the parasite (asterisks). In (b), a giant LB is seen close to the nucleus in an irradiated-infected rat. (c) LB diameter variation in different groups. A significant increase of LB occurred in infected alone compared to uninfected and in irradiated-infected compared to infected alone groups ($P < 0.05$). Before infection, rats were irradiated or not and heart samples processed for transmission electron microscopy at day 12 of infection. Scale bar, 1.0 μm (a); 600 nm (b). Reprinted from [29] with permission.

The lipid body-phagosome association has also been observed in cells infected with other pathogens such as *M. bovis* BCG [31], *M. tuberculosis* [39], and *Chlamydia trachomatis* [40]. It remains to be established if it represents a strategy for pathogen replication or if has implications for pathogen outcome.

3. Lipid Body and Inflammation

As noted, accumulation of lipid bodies has been observed in the cytoplasm of activated cells associated with varied inflammatory and infectious conditions, both in clinical and experimental situations [23, 26, 31, 35, 36, 41, 42].

Lipid bodies are recognized sites for localization of arachidonic acid, the precursor for the synthesis of inflammatory mediators (eicosanoids) and eicosanoid-forming enzymes such as cyclooxygenases (COX) and lipoxygenases (LO) (reviewed in [12, 43]). The production of eicosanoids has been demonstrated during *T. cruzi* infection and both leukotrienes and prostaglandins seem to play a role in the pathogenesis of Chagas' disease. Macrophages, the first line of defense, are important sources for prostaglandin E_2 (PGE_2) production during *T. cruzi* infection [44–46] and

parasite-induced lipid body formation within macrophages is accompanied by enhanced COX-2 expression [35]. In fact, by using fluorescence microscopy, it was demonstrated that *T. cruzi*-infected macrophages were found to be positive COX-2. The immunostaining appeared punctuate throughout the cytoplasm, suggesting that COX-2 may be localized within lipid bodies, in addition to the conventional perinuclear membrane localization. Colocalization of COX-2 and adipose differentiation-related protein (ADRP), a recognized marker for lipid bodies [47, 48], confirmed the presence of this enzyme within lipid bodies [35].

The increased formation of lipid bodies within inflammatory macrophages is accompanied by significant production of the PGE_2. The highest numbers of lipid bodies induced by the *T. cruzi* infection in inflammatory macrophages occurred in parallel to the highest production of PGE_2 [11] (Figure 5). This increase was documented at days 6 (fourfold) and 12 (sixfold) after infection in rats [11] (Figure 5). In murine model, the increased PGE_2 production derived from lipid bodies was rapidly observed at 24 h of infection (fourfold), through a TLR2-dependent manner [35].

Prostaglandins down modulate a number of macrophage functions. Prostaglandins lead to reduced proinflammatory cytokine secretion, decreased antigen presentation, and diminished production of free radicals in these cells [49–52]. Studies have been demonstrating that prostaglandins are potent inhibitors of tumor necrosis factor-alpha (TNF-α) synthesis and act as deactivators of macrophage trypanocidal function [46, 53]. Moreover, high levels of PGE_2 favor *T. cruzi* replication and the treatment of the infected mice with nonsteroidal anti-inflammatory drugs (NSAIDs), inhibitors of COX-2 enzyme, significantly reduces parasite replication [35, 44, 54]. Interestingly, addition of exogenous PGE_2 is also able to increase replication of the parasite *Leishmania amazonensis* in macrophages, indicating that PGE_2 increases intracellular load of this pathogen in susceptible mice [55].

In contrast to inflammatory macrophages, peripheral blood monocytes from *T. cruzi*-infected animals show low number of lipid bodies [11]. The maturation of peripheral blood monocytes to tissue macrophages followed by activation of these cells is likely involved in lipid body formation and eicosanoid release during Chagas'disease [11].

Eicosanoids may also be directly observed within lipid bodies. By using a newly developed strategy, the Eicosacell methodology [56], which directly detects the eicosanoid synthesis *in situ*, intracellular sites of newly formed PGE_2 colocalized with ADRP-labeled lipid bodies, confirming that lipid bodies are sites of compartmentalization of PGE_2 synthesis during *T. cruzi* infection [35].

During the *T. cruzi* infection, the role of prostaglandins in the outcome of the parasite is still a matter of debate. It has been shown that the widely used nonsteroidal anti-inflammatory drugs (NSAIDs), aspirin (an inhibitor of both constitutive COX-1 and inducible COX-2) [57] and indomethacin (a preferential inhibitor of COX-1) [57], inhibit PGE_2 synthesis and are able to control parasitaemia in susceptible mice [44]. Likewise, aspirin and NS-398 (COX-2 inhibitor) [58]) were able to modulate lipid body formation and consequently to inhibit the PGE_2 production and parasite growth in macrophages [35]. These data support the concept that *T. cruzi* induces and exploits host-derived lipid bodies to extend and maintain its own survival. Moreover, pharmacologic intervention of lipid body biogenesis inhibits *T. cruzi* survival and replication in macrophages, and therefore these organelles may act as potentially targets for therapy during the acute phase of Chagas' disease [35].

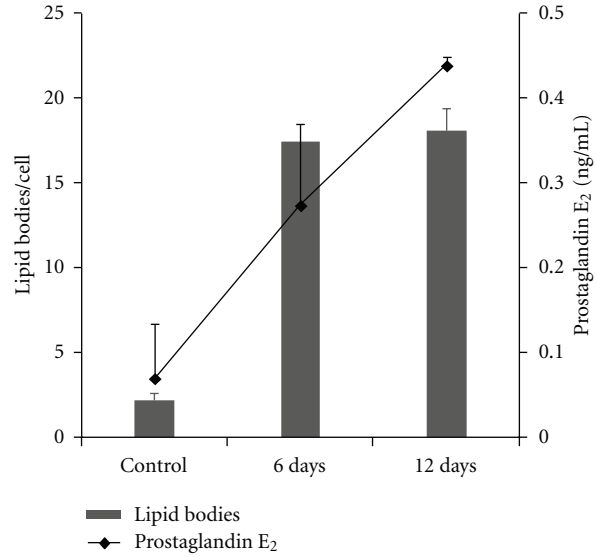

Figure 5: *Trypanosoma cruzi* infection induces concomitant lipid body formation and prostaglandin E_2 (PGE_2) synthesis. Associations between number of lipid bodies (bars) and prostaglandin E_2 (PGE_2) peritoneal levels (line) in rats at day 6 or 12 of infection with *T. cruzi* and in uninfected controls. At both days, the lipid body numbers were significantly increased ($P < 0.05$) in parallel to an accentuated increase of PGE_2 synthesis. Data are expressed as means ± SEM. Reprinted from [11] with permission.

4. Lipid Body and Apoptosis during *T. cruzi* Infection

Apoptosis of host cells, mostly lymphocytes and cardiomyocytes, has been identified during the *T. cruzi* infection in both humans and experimental models and seem to play an important immune regulatory role in this and other parasitic infections [44, 59, 60].

Uptake of apoptotic bodies, a process termed efferocytosis, is able to impact on host inflammatory mediator production and susceptibility to infection [44, 60, 61]. It was recently demonstrated that efferocytosis may affect lipid body formation and PGE_2 synthesis during the *T. cruzi* infection [35] (Figure 6). These authors showed that the uptake of apoptotic cells, but not living or necrotic cells by cultured macrophages, triggers lipid body formation in the absence of infection. However, when infected macrophages are exposed to apoptotic cells, the efferocytosis process amplifies the effects of the parasite on lipid body formation leading to a higher lipid body accumulation compared to noninfected cells exposed to apoptotic bodies (Figure 6).

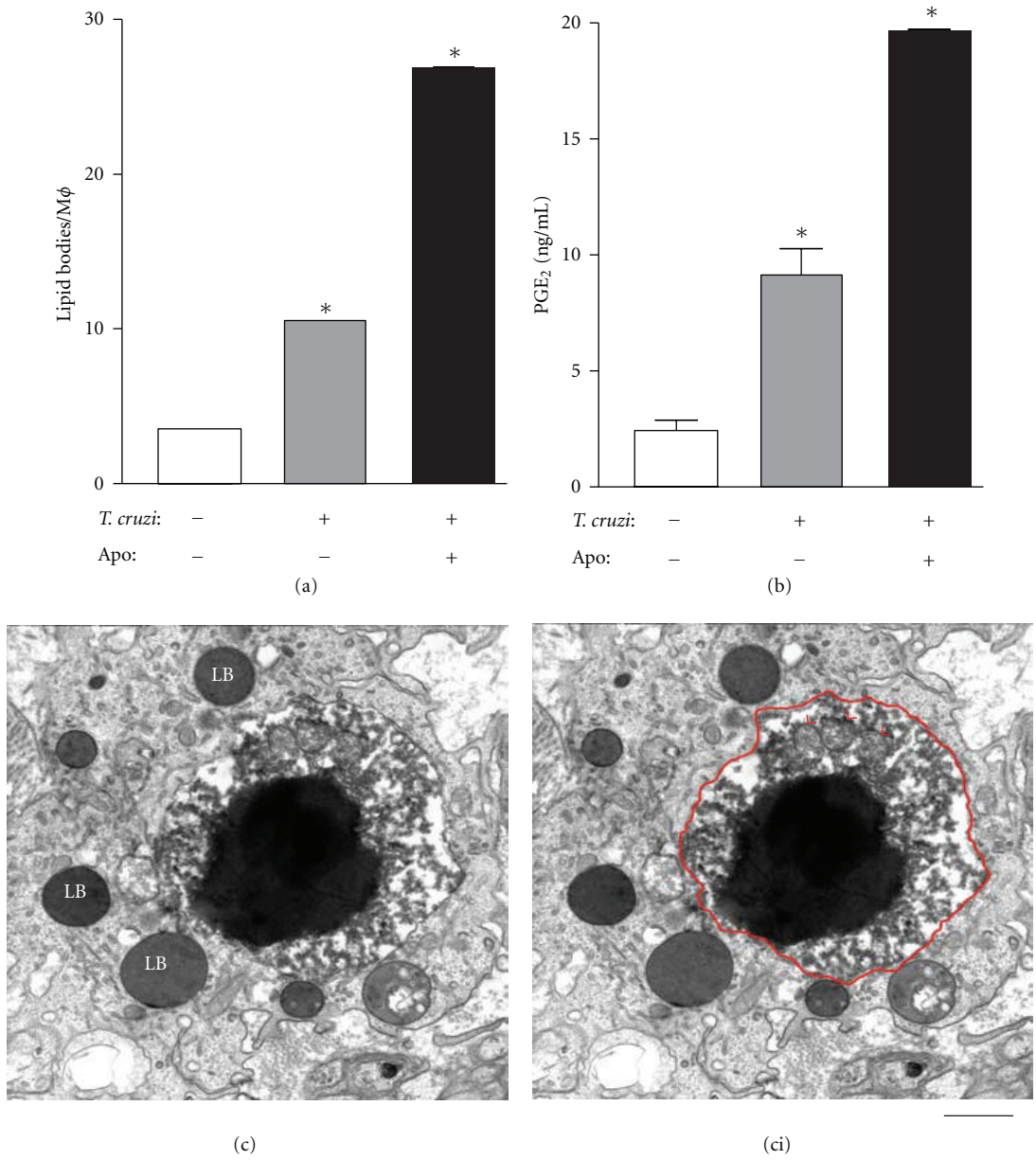

Figure 6: Uptake of apoptotic cells by macrophages induces lipid body formation and PGE_2 synthesis during *Trypanosoma cruzi* infection. LB formation (a) and prostaglandin E_2(PGE_2) synthesis (b) by mice macrophages infected *in vitro* with *T. cruzi* alone or co-cultured with apoptotic cells for 24 hours. Each bar represents the mean ± standard error of the mean (SEM) from 50 consecutively counted macrophages from at least 4 independent pools of 3 animals each. Statistically significant ($P \# 0.05$) differences between control and infected or stimulated groups are indicated by asterisks; Mϕ, macrophages. In (c), a large typical apoptotic body (outlined in red in (ci)) showing very condensed, electron-dense nuclear chromatin and degenerating mitochondria (red arrowheads) is seen within a heart macrophage of a rat with 12 days of infection. Note that the apoptotic body is surrounded by several lipid bodies with distinct electron densities. Rats were infected with the Y strain of *T. cruzi* and the myocardium processed for transmission electron microscopy at day 12 of the acute infection. Reprinted from [35] with permission. Scale bar, 1 μm.

Consistent with the lipid body function in inflammation, as discussed in Section 3, the uptake of apoptotic cells by both infected and noninfected macrophages induces increased lipid body-derived PGE_2 synthesis. Interestingly, 24 h after the uptake of apoptotic cells by noninfected macrophages, newly formed lipid bodies show *in situ* both COX-2 and PGE_2, similarly to infected cells [35].

Freire de Lima and colleagues [44] demonstrated that the recognition of apoptotic cells by the $\alpha_v\beta_3$ integrin (vitronectin receptor) is decisive for apoptotic-cell cytoadherence and the induction of both PGE_2 and transforming growth factor beta (TGF-β) release during *T. cruzi* infections. The involvement of $\alpha_v\beta_3$ integrin on lipid body formation and parasite replication induced by apoptotic cell uptake was

also evaluated utilizing flavoridin, a desintegrin that blocks binding of integrins $\alpha_v\beta_3$ [62]. Flavoridin blocked lipid body formation and COX-2 expression, induced by incubation of macrophages with apoptotic cells, indicating that the engagement and activation of $\alpha_v\beta_3$ is sufficient to trigger lipid body biogenesis, COX-2 expression, and enhanced PGE_2 synthesis in macrophages [35].

Distinct works have been demonstrating that TGF-β is consistently produced during the *T. cruzi* infection [35, 63, 64]. Moreover, as noted, the interaction of macrophages with apoptotic cells leads macrophages to produce TGF-β and also renders phagocytic cells more permissive to *T. cruzi* infection [35, 63–65]. A recent report showed that TGF-β induces lipid body formation, affecting in turn the PGE_2 release, and making phagocytic cells more permissive to *T. cruzi* infection [35]. In fact, in conjunction with increased lipid body formation and PGE_2 production, infected macrophages presented exacerbated parasite replication when cocultured with apoptotic cells for 24 hours [35]. The capacity of other cytokines to modulate lipid body formation in macrophages during the *T. cruzi* infection remains to be defined.

As mentioned in Section 3, aspirin and NS-398 inhibit cyclooxygenase production. Likewise, these drugs are able to inhibit lipid body formation in infected macrophages in the presence or absence of apoptotic cells, suppresses apoptotic cell effects on lipid body-derived PGE_2 production, and reverses the effects of apoptotic cells on parasite replication [35]. Accordingly, the fatty acid synthase inhibitor C75 significantly inhibited lipid body formation induced by *T. cruzi*, with or without the presence of apoptotic cells. Strikingly, it was demonstrated that the treatment with C75, in parallel to lipid body inhibition, reversed the parasite replication induced by apoptotic cells [35]. In summary, macrophage lipid bodies formed during *T. cruzi* infection in association with apoptotic cell stimulation directly impact the capacity of macrophages to produce increased amounts of PGE_2, which may have impact on the ability of the host to control the infection.

5. Final Remarks

In recent years, the association of lipid bodies, morphologically distinct organelles, with many potential roles in cells, in both health and disease, has brought special attention to them. Lipid body accumulation has been documented in varied inflammatory situations, in both experimental and human conditions [12, 25, 66].

Research over the last decade has identified an important formation of lipid bodies within inflammatory macrophages in response to the *T. cruzi*-host interaction. Newly formed lipid bodies during Chagas' disease and other inflammatory diseases are notable for their capacity to synthesize inflammatory mediators, such as PGE_2 and for expressing enzymes linked to this synthesis, such as COX-2 [12, 21]. Lipid bodies elicited by *T. cruzi* and other pathogens are now recognized not only as inflammatory organelles and structural markers of pathogen-induced cell activation, but also as organelles able to modulate host cell processes [11, 29, 35]. For example, a recent work supports the concept that *T. cruzi* induces and exploits host-derived lipid bodies to extend and maintain its own survival [35]. However, much remains to be learned. More work will be needed to understand the influence of lipid bodies on the host cell physiology and if these organelles have a major role in the destruction or intracellular survival of the parasite. The increased knowledge of lipid bodies in pathogenic mechanisms of infections may not only contribute to the understanding of pathogen-host interactions but may also identify new targets for intervention.

Acknowledgments

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Research Article

oxLDL Downregulates the Dendritic Cell Homing Factors CCR7 and CCL21

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Introduction. Dendritic cells (DCs) and oxLDL play an important role in the atherosclerotic process with DCs accumulating in the plaques during plaque progression. Our aim was to investigate the role of oxLDL in the modulation of the DC homing-receptor CCR7 and endothelial-ligand CCL21. *Methods and Results*. The expression of the DC homing-receptor CCR7 and its endothelial-ligand CCL21 was examined on atherosclerotic carotic plaques of 47 patients via qRT-PCR and immunofluorescence. *In vitro*, we studied the expression of CCR7 on DCs and CCL21 on human microvascular endothelial cells (HMECs) in response to oxLDL. CCL21- and CCR7-mRNA levels were significantly downregulated in atherosclerotic plaques versus non-atherosclerotic controls [90% for CCL21 and 81% for CCR7 ($P < 0.01$)]. *In vitro*, oxLDL reduced CCR7 mRNA levels on DCs by 30% and protein levels by 46%. Furthermore, mRNA expression of CCL21 was significantly reduced by 50% ($P < 0.05$) and protein expression by 24% in HMECs by oxLDL ($P < 0.05$). *Conclusions*. The accumulation of DCs in atherosclerotic plaques appears to be related to a downregulation of chemokines and their ligands, which are known to regulate DC migration. oxLDL induces an *in vitro* downregulation of CCR7 and CCL21, which may play a role in the reduction of DC migration from the plaques.

1. Introduction

Atherosclerosis is a dynamic inflammatory disease [1, 2] in which autoantigenes, such as oxidized low-density protein (oxLDL), play an essential role. Dendritic cells (DCs) are key regulatory antigen-presenting cells (APCs) that induce inflammatory processes. DCs can be detected in the vascular inflammatory environment, from endothelial dysfunction to consecutive plaque formation and rupture [3–5]. DCs are the most potent APCs and are highly specialized to prime naive T-cells [6]. Interestingly, mature DC-specific markers, for example, CD83, accumulate during plaque progression. It has been shown previously that the presence of CD83 is more than two fold, higher in symptomatic compared to asymptomatic patients [7].

DCs, after entering the vascular tissue, screen the environment for potential antibodies [2]. After processing the antigens, a maturation process is initiated and immunomodulatory receptors, such as the CD83-receptor (important for T-cell-stimulation), are upregulated [8]. In a previous study, we found a CD83 upregulation on DCs by proatherosclerotic stimuli like oxLDL and asymmetric dimethylarginine (ADMA) [9]. Beside CD83, DC induces CCR7 expression during maturation, which acts as a receptor for constitutively expressed CCL21 and CCL19 [10, 11]. In analogy to DC accumulation and maturation, activated CCR7 expressing

T-cells are trapped especially in the plaque shoulders [12, 13] where mature DCs are forming clusters with T cells [13].

Several studies have demonstrated the important link between DC/T-cell recruitment and CCR7 expression [12–14]. The increase in CCR7 receptors induces homing of mature DCs and T-cells to the lymph nodes through CCL21/CCL19 expressing lymph vessels [2, 11, 12].

High plasma concentrations of oxLDL and their appearance in atherosclerotic lesions are of utmost importance in the pathogenesis of atherosclerosis [15].

The present study focuses on the influence of oxLDL on the DC-related chemokine receptors CCR7, CCL19, and CCL21. We characterized the DC-specific chemokine-ligand expression in human atherosclerotic carotid artery plaques. Furthermore, we investigated the impact of oxLDL on the DC receptor CCR7 expression and its ligands CCL-21 on human microvascular endothelial cells (HMECs).

2. Materials and Methods

2.1. In Vivo

2.1.1. Study Population. All investigations were approved by the institutional review board and the ethics committee of the Ludwig-Maximilians University of Munich. Informed consent was obtained from all patients. The investigation conforms to the principles outlined in the declaration of Helsinki. Between September 2006 and May 2008, carotid endarterectomy (CEA) was performed in 47 patients. The indications for CEA were based on carotid duplex sonography: stenosis of the internal carotid artery of more than 70% for symptomatic patients and more than 85% for asymptomatic patients. For comparison, we collected 10 blood samples from healthy age-matched men without clinically manifested atherosclerosis and 14 pieces of aortic tissue with no visible atherosclerotic lesions.

2.1.2. RNA Isolation from Atherosclerotic Plaque for Real-Time PCR. 100 mg plaque tissue was homogenized in 1 mL QIAzol Lysis. 50 pg/tube RNA (PBMCs) was used. First-strand cDNA synthesis and PCR were performed using Omniscript from Qiagen (Hilden, Germany). The two-step quantitative real-time PCR (rt-PCR) system was applied according to the manufacturers' instructions. The quantitative real-time PCR system provides optimal performance with SYBR Green primers (Qiagen: Hilden, Germany). Rt-PCR was performed in the ABI PRISMTM 7700 System (Applied Biosystems, Germany). Data analysis was performed using the delta-delta-Ct method as described previously [16]. Primer sequences for the amplified fragments were GAPDH: 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′/5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′; CCR7: 5′-TGG AGG CCT TTA TCA CCA TC-3′/5′-TGT AGG GCA GCT GGA AGA CT-3′; CCL19: 5′-CTG TGA CCC AGA AAC CCA TC-3′/5′-GCT TCA TCT TGG CTG AGG TC-3′; CCL21: 5′-CCC AGC TAT CCT GTT CTT GC-3′/5′-TCA GTC CTC TTG CAG CCT TT-3′; CD4: 5′-AGG AAG TGA ACC TGG TGG TG-3′/5′-CTC AGC AGA CAC TGC CAC AT-3′.

2.1.3. Immunofluorescence Analysis of CCL21 Expression in Human Plaque and Aortic Tissue. Frozen sections (10 μm) were prepared from human aorta and plaque material of the internal carotid artery. After fixation in methanol/ethanolic acid for 1.5 min at −20°C, the sections were washed immediately 3 times for 3 min in PBS. Unspecific binding was blocked with a 2% BSA/0.2% Tween 20 solution for 1 h and followed by 3 washing steps in PBS. To visualize nucleoli we used DAPI (4′, 6′Diamidino-2′phenylindole, Merck KGaA, Germany) in a final concentration of 1 μg/mL. After another washing step the sections were incubated with a fluorescent labelled anti-human CCL21/6Ckine antibody (5 μg/mL, R&D Systems, Inc., Germany).

To determine the localization of the CCL21 expressing structure (CCL21/6Ckine antibody 5 μg/mL, R&D Systems, Inc., Germany), the sections were also incubated with a Cy3-labeled anti-PECAM-1 antibody (5 μg/mL, AbD Serotec, Germany), which demarcates the endothelia cells in the vessel wall. Immunofluorescence analysis of CCL21 expression was investigated by confocal microscopy (LSM 510 META, Zeiss, Plan-Neofluar 40x/1.30 oil objective, Germany) and quantified by LSM Image Browser 4.2 (Zeiss, Germany).

2.1.4. Immunofluorescence Analysis of CCR-7/CD4- and CCR-7/CD83-Coexpression in Human Plaque and Aortic Tissue. The sections were incubated with an FITC-labelled anti-human CCR7 antibody (1 : 100 concentration, R&D Systems, Inc., Germany) for 1 h, followed by 3 washing steps in PBS.

The sections were incubated with an Atto594-labeled anti-CD4 antibody (10 μg/mL, AbD Serotec, Germany) or Atto594-labelled anti-CD83 (10 μg/mL, AbD Serotec, Germany) for 1 h, which represents the T-lymphocytes (CD4) or mature DC (CD83). The immunofluorescence analysis was investigated by confocal microscopy (LSM 510 META, Zeiss, Plan-Neofluar 40x/1.30 oil objective, Germany).

2.1.5. ELISA for oxLDL and CCL21. Plasma tubes were centrifuged and the plasma was fractionated and frozen at −80°C.

oxLDL levels were examined by using a cytokine-specific ELISA kit according to the manufacturers' instructions (ImmunDiagnostik, Bensheim, Germany) [17]. Before analysis, samples were treated as described above. In brief, after washing with wash buffer several times, standard samples and controls were added and incubated for 4 hours at room temperature on a horizontal mixer. Thereafter, a washing conjugate was added and incubated for 1 h as described before. The substrate was added and incubated for 25 minutes in the dark. Stop-solution was added and immediately analyzed by an ELISA-reader at 450 nm.

Detection of *CCL21 in serum* was also performed by a quantitative sandwich enzyme immunoassay technique, according to the manufacturers' instructions (R&D Systems, Germany).

2.2. In Vitro

2.2.1. Generation of Monocyte-Derived Dendritic Cells (DCs). Mononuclear cells were isolated from 100 mL of peripheral blood of a healthy human donor by a Ficoll density gradient according to the protocol by Boyum [18]. The purity of the monocyte culture was enhanced up to 97% by adhesion on γ-globulin coated plates. DCs were obtained from the monocyte culture according to the modified protocol by Romani et al. [19] as described in detail in our recent publication [9, 17].

2.2.2. Endothelial Cells (ECs). All experiments were performed with human microvascular endothelial cells (HMECs-1), generously provided by the Center for Disease Control and Prevention and the National Center for Infectious Disease (Atlanta, USA). Cells were cultured in MCDB-131 (without phenol red; cc pro, Neustadt/W., Germany) supplemented with 10% fetal calf serum (FCS; PAA, Pasching, Austria), 2 mM L-Glutamine (Biochrom AG, Berlin, Germany), 1 μg/mL hydrocortisone, and 10 ng/mL epidermal growth factor (Sigma, Taufkirchen, Germany). The cells were used at least 10 days after thawing and for no more than 20 passages [20].

2.2.3. LDL Oxidation. LDL (density = 1.019 to 1.063 g/mL) was isolated from human plasma of normolipidemic healthy volunteers by sequential ultracentrifugation as described and stored in PBS containing 2 mmol/L EDTA. Shortly before oxidation, the EDTA was removed from LDL by passing the lipoprotein through a PD 10 column (Pharmacia, Austria). LDL was oxidized in Ham's F-10 medium by exposure to 5 μmol/L $CuSO_4$ at 37°C for 30 h [11, 21]. All preparations were dialyzed before they were added to the cultured cells. This reflects oxLDL concentrations in the plaque and has been used in previous studies [5, 22, 23]. In addition, several other studies have shown that an oxLDL concentration of (10 μg/mL) is not toxic to the cultured cells [9, 24, 25]

2.2.4. Total RNA Isolation and Real-Time PCR. For isolation of mRNA from HMECs-1 and DCs, the total RNA isolation RNAeasy Mini Kit from Qiagen (Hilden, Germany) was used according to the instructions provided by the manufacturer. 50 pg/tube RNA (PBMC) was used. First-strand cDNA synthesis and PCR were performed using Omniscript from Qiagen (Hilden, Germany). The two-step quantitative RT-PCR system was applied according to the manufacturers' instructions and as described above [16].

2.2.5. Flow-Cytometric Analysis of DCs. Cells were incubated with antibodies according to the manufacturers' instructions. Fixed cells were analyzed on an FACS Calibur cytometer (Becton Dickinson). Antibodies were matched to iso-type-controls (Mouse-γ2a-(FITC)/-γ1(PE)-Fastimmune; BD; USA). To verify the purity of our DC-culture, we used CD3 (BD, USA) and CD20 (BD, USA) to rule out a T-cell and B-cell contamination (<5%). DCs were characterized by low CD14 expression (BD, USA) and high expression of CD80 (BD, USA), CD86 (BD, USA), and HLA-DR (BD, USA), as described previously [26].

At day five, we incubated the DCs with oxLDL (10 μg/mL) for 24 hours. Expression analysis of the chemok-ine receptor CCR7 (R&D, USA) and CD83 (BioLegend, USA) on human DCs were performed using flow-cytometer analysis [16, 17].

2.2.6. Immunofluorescence of CCL21 on HMECs-1. HMECs-1 were cultivated on chamber slides and stimulated with 10 μg/mL oxLDL for 48 h. The monoclonal antibody for CCL21/6Ckine (R&D, USA) was labeled with Alexa 488 using the Alexa Fluor Protein Labeling Kit (Invitrogen, life technologies, USA) according to the manufacturers' instructions. HMECs-1 were incubated with the labeled antibody (concentration 1 : 20) for 30 minutes. Thereafter, Vybrant DiD (Invitrogen, life technologies, USA) was used as a marker of the plasma membrane. CCL21-labeled HMECs-1 were investigated by confocal microscopy (Zeiss LSM 510 Meta, Plan-Apochromat 63x/1.40 oil objective) and quantified by LSM Image Browser 4.2 (Zeiss, Germany).

2.2.7. Statistical Analysis. Data is presented as ± standard-deviation of the mean (SDM). The Kolmogorov-Smirnov test was used to determine whether or not the data was normally distributed. If the data was normally distributed, the unpaired t-test was used to compare the two groups. Data that was not normally distributed was compared using the Wilcoxon signed Rank Test. Differences between means were considered significant with $P < 0.05$ and highly significant with $P < 0.01$. All *in-vitro* experiments were repeated at least eight times with different cells and lipoprotein preparations. SPSS (version 16, Leibniz Rechenzentrum Munich) was used for statistical analysis.

3. Results and Discussion

3.1. In Vivo

3.1.1. Study Population. Carotid plaque tissue and blood samples were collected from 47 patients undergoing CEA, of which 77% were male and, on average, 70 ± 8 years of age. 66% had asymptomatic disease, whereas and the rest showed cerebral ischemic complications. The different risk profiles and medication are summarized in Table 1.

Serum for the PBMCs in the control group was collected from 10 healthy age-matched patients. 60% were of male gender with an average age of 68 ± 4 years. None had cerebral ischemic complications or clinical manifest atherosclerosis. Concerning the risk-profile, 20% had insulin-dependent diabetes mellitus, 80% hypertension, and 10% were smokers. Concerning the medication, 10% took thrombocyte aggregation inhibitors, 80% statins, and 60% had a β-blocker (Table 1).

Aortic tissue in the control group was collected from 14 patients who underwent valve replacement and had no sign of aortic atherosclerosis. 71% were of male gender with an average age of 64 ± 5 years. 14% had a cerebral

Table 1: Clinical characteristics of the patients and controls. Clinical characteristics including age, sex, cardiovascular risk factors, relevant medication, oxLDL and CCL21 of patients who received CEA and healthy controls without clinical history of manifest atherosclerosis.

	CEA-patients ($n = 47$)	healthy controls ($n = 10$)
Age (years)	70 ± 8	68 ± 4
men % (n)	77 (36)	60 (6)
TIA/CI % (n)	32 (15)	0 (0)
ID DM % (n)	26 (12)	20 (2)
Hypertension % (n)	91.5 (43)	80 (8)
Hyperlipidemia % (n)	85 (40)	80 (8)
Nicotine abusus % (n)	38 (18)	10 (1)
ASS/Clopidogrel % (n)	91.5 (43)	10 (1)
β-blocker % (n)	60 (28)	60 (6)
Statins % (n)	79 (37)	80 (8)
oxLDL (ng/mL)	207.3 ± 240.2	99.4 ± 20.3
CCL21 (pg/mL)	1335.1 ± 771.5	961.2 ± 210.9

ischemic event. Concerning the risk profile, 28% had insulin-dependent diabetes mellitus, 85% hypertension, and 35% were smokers. With respect to medication, 43% took thrombocyte aggregation inhibitors, 78% statins, and 43% had a β-blocker.

3.1.2. CCL21/CCL19 and CD83/CCR7 and CD4 mRNA Analysis of the Atherosclerotic Plaque. In line with previous investigations, we detected that the median CD83 mRNA levels in plaque tissue from CEA-group were nearly twice as high as in healthy aortic tissue ($P < 0.01$), demonstrating the augmented presence of mature DCs in the atherosclerotic tissue [7, 27, 28]. In a subgroup analysis, we further found that CD83 levels in men were significantly higher (+120%; $P < 0.05$) compared to women. CD4 mRNA levels were even 23 ± 11 fold higher in healthy aortic tissue ($P < 0.01$; data not shown).

CCR7, normally coexpressed on mature DCs and T-cells, was 81% lower in the plaque compared to compared to healthy aortic tissue ($P < 0.01$) without any gender specific differences.

Further analysis of CCL19 revealed that its transcripts were downregulated by 99% ($P < 0.01$). CCL21 was found 90% lower in the plaque ($P < 0.01$) compared to healthy aortic tissue (Figure 1).

Furthermore CCL21 was also found lower in the symptomatic patients (58%; $P < 0.05$) compared to asymptomatic patients from the CEA group.

3.1.3. Immunofluorescence Analysis of CCL21 Expression in Human Plaque and Aortic Tissue. To confirm the PCR results on the protein level, we performed a semiquantitative immunofluorescence analysis on histological slides of human aortic and plaque tissue.

To analyze the location of CCL21 expressing cells, we stained the endothelial cells (α-PECAM-1-Cy3) in the aortic tissue section. This revealed a colocalization of the CCL21 and the vascular wall of the vasa vasorum (Figure 2(a)).

In every single healthy aortic tissue slide ($n = 6$), we could detect higher CCL21 expression via an increased fluorescence signal signal from the binding fluorescent antibody than in the plaque tissue ($n = 15$). An example is shown ($n = 15$; in Figure 2(b)).

3.1.4. Immunofluorescence Analysis of CCR-7/CD4 and CCR-7/CD83 Coexpression in Human Plaque and Aortic Tissue. To further investigate differences of the CCR7 level on T-lymphocytes (CD4 positive) and mature DC (CD83 positive) in human tissue, immunofluorescence analysis was used. By double staining, we could detect a decreased signal of CCR7 on CD4-positive cells within the plaque tissue (Figure 2(c); lower panel) compared with control aortic tissue (Figure 2(c); upper panel). Also, CD83-expressing cells within the plaque show a decreased CCR7 signal (Figure 2(d); lower panel) compared to the aortic tissue which correlates with the PCR results.

3.1.5. Circulating oxLDL and CCL21 Concentrations. For oxLDL, we found a 52.1% ($P < 0.05$) higher concentration in the plaque group compared to the control group (Table 1). oxLDL serum concentrations showed no correlation with CCL21/CCL19 or CCR7 expressions in plaques (Table 1).

For CCL21, we found a 28% ($P < 0.01$) higher serum concentration in the CEA group compared to the controls (Table 1).

3.2. In Vitro Cell Experiments

3.2.1. CCR7 and CCL21/CCL19 Expression in DCs and HMECs-1. After stimulation with oxLDL, we found a reduction of the mRNA levels of CCR7 (DCs) by 30% using RT-PCR ($n = 8$; $P < 0.05$). This correlated with a reduction in protein expression by 46% ($n = 8$; 25.7 ± 1.06% versus control 47.6 ± 19.3% positive cells; $P < 0.05$; Figure 3). In contrast, protein expression of CD83 was significantly upregulated by 10 μg/mL oxLDL, as shown in Figure 3 (60%, 46.9±11.3 versus control 29.3±17.7% positive cells; $P < 0.05$; Figure 2) by 10 μg/mL oxLDL (Figure 3).

For HMECs-1, stimulation with oxLDL led to a significant reduction of CCL21 mRNA expression (50%; $\Delta\Delta$ct = 0.5; $P < 0.05$, Figure 4(a)). These results correspond with the results of the immunofluorescence analysis, where we found a 24% down-regulation of CCL21 receptor expression (n = 9; 34.00 ± 8.53 versus control 44.54 ± 6.23 intensity/mm^2; $P < 0.05$; Figures 4(b) and 4(c). oxLDL had no significant impact on protein or mRNA expression of CCL19 (Figure 4(a)).

4. Discussion

The main findings obtained from our study are (1) The DC-chemokine receptor CCR7 and its ligands CCL21/CCL19 are significantly downregulated in atherosclerotic plaques;

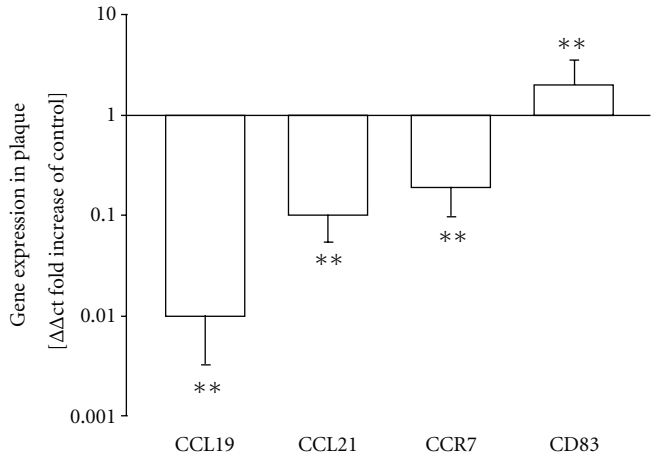
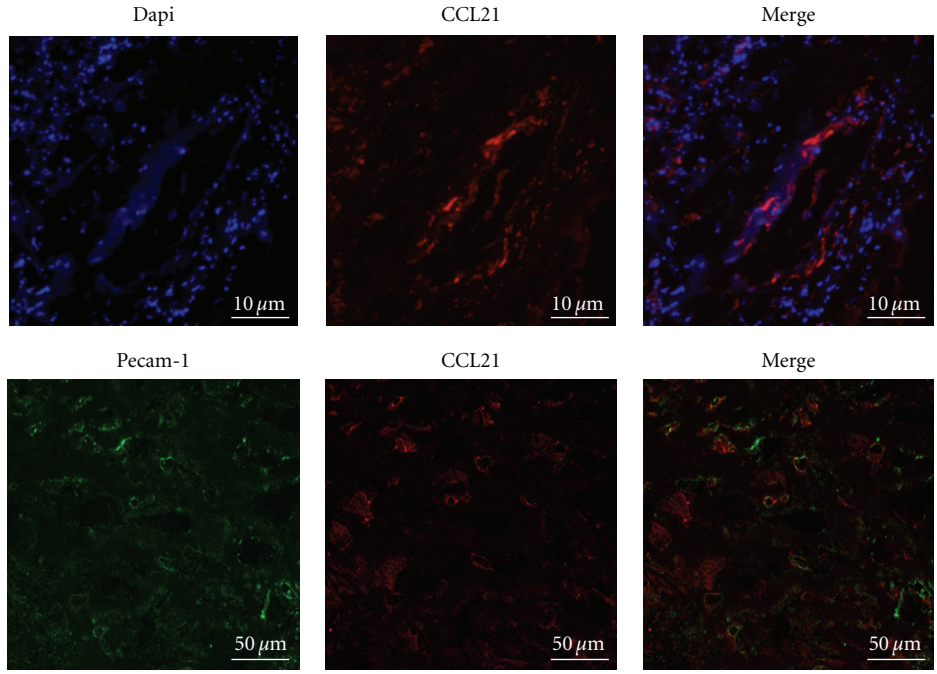
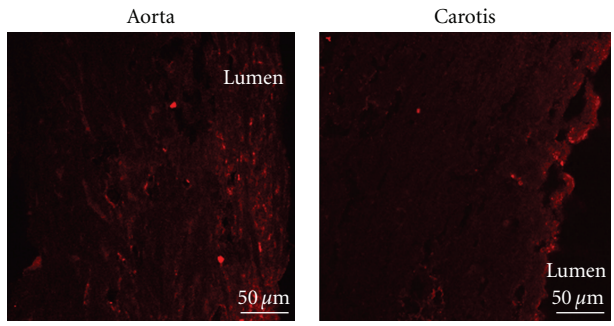


Figure 1: mRNA expression of CCL21/19, CCR7, and CD83 in vascular plaques compared to aortic tissue without plaque detection. *In vivo* changes (relative fold increases) in mRNA expression of CCL19, CCL21, CCR7, and CD83 in carotid plaques compared to healthy aortic material. $^{*}P < 0.05$, $^{**}P < 0.01$, ($n = 47$) versus aortic tissue ($n = 14$).

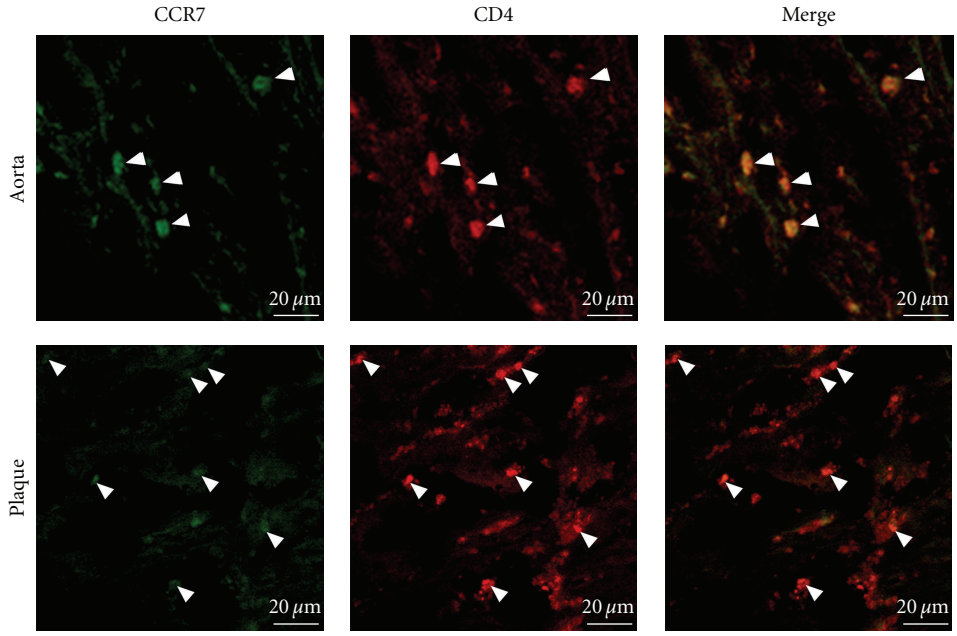


(a) Analysis of the CCL21, expressing structures in human aortic tissue

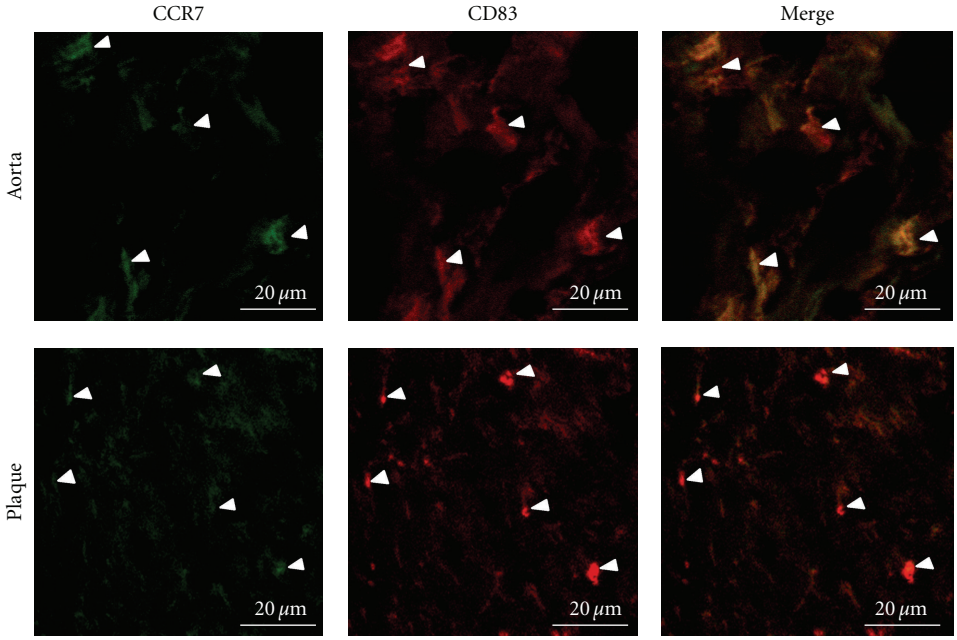


(b) Localisation of CCL21, positive cells in aortic tissue and carotis plaque

Figure 2: Continued.



(c) Analysis of the coexpression of CCR7 and CD4



(d) Analysis of the coexpression of CCR7 and CD83

Figure 2: Protein expression of CCL21 and CCR7 on carotid plaque and aortic tissue. (a) Analysis of the CCL21 expressing structures in human aortic tissue. In the upper part the blue staining represents the cell nuclei (DAPI) and the red staining represents CCL21, positive structures (α-CCL21/6Ckine-Alexa488, pseudocolored). Scale bar 10 μm. To examine the vessel wall as CCL21 expressing structures (lower part) in human aorta tissue endothelial cells were stained with a PECAM-1 antibody (green, α-PECAM-1-Cy3, pseudocolored). The red staining represents CCL21 (α-CCL21/6Ckine-Atto594). The merged picture shows a co-localization of CCL21 and PECAM-1 in the vessel wall of the human aorta. Scale bar 50 μm. (b) The representative pictures show differences in the expression level of CCL21 (red, α-CCL21/6Ckine-Atto594) in aorta and carotid plaque tissue. A decreased CCL21-protein expression in carotid plaque compared to aorta tissue was shown by immunofluorescence microscopy. CCL21 is primarily expressed by the vasa vasorum of the vessel. Scale bar 50 μm. (c) A decreased CCR7 expression (green, α-CCR7-FITC) on CD4 positive cells (red, α-CD4-Atto594) was detectable in plaque tissue compared to aorta tissue. Arrows indicate the cells. Scale bar 20 μm. (d) Also a decreased CCR7 expression (green, α-CCR7-FITC) on CD83, positive cells (red, α-CD83-Atto5994) in aorta and plaque tissue is shown in the representative pictures. Arrows indicate the cells. Scale bar 20 μm.

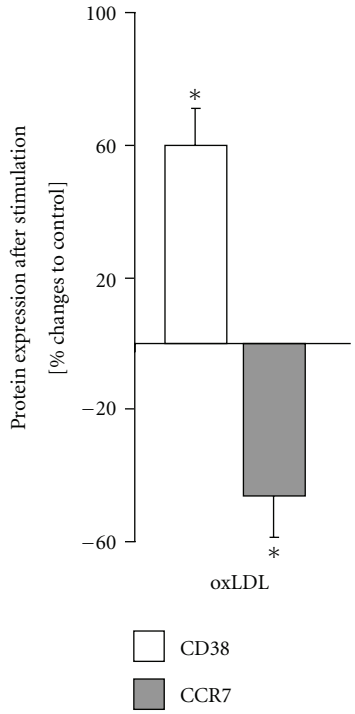


Figure 3: Protein expression of CD83 and CCR7 on DCs after stimulation with oxLDL. An increase of the expression of CD83 and a decrease of the expression of CCR7on DCs after stimulation with oxLDL (10 μg/mL) was observed. Positive cells were measured by flow cytometry and the percent (%) changes were plotted in the figure. $^*P < 0.05$, ($n = 8$) versus unstimulated controls.

(2) Circulating CCL21 levels are upregulated in serum from atherosclerotic patients; (3) oxLDL impairs CCR7 expression in DCs and CCL21 expression in microvascular ECs.

Our data support the concept that modulation of chemokine receptors (mediated, e.g., by oxLDL) in the plaque may trigger retention of DCs, thereby impeding the vascular innate and adaptive immunity [29, 30]. Accordingly, Angeli et al. found that oxLDL and other lipid mediators are jointly responsible for trapping of DCs in the vascular wall [29]. In our *in vivo* study, oxLDL serum concentrations showed no correlation with CCL21/CCL19 or CCR7 expression in plaques. However, CCR7 and CCL21/CCL19 were downregulated in atherosclerotic plaques compared to nonatherosclerotic aortic tissue. This discrepancy may be explained by the fact that oxLDL is unstable in serum but accumulates in the subintimal space over time. Hereby, it reaches much 70 fold higher subintimal concentrations as compared to serum levels [31]. This fact especially appeases to monocytes rich plaques [31].

We found CCR7 expression to be downregulated after 24 hours of stimulation with oxLDL. Alongside, CCL21 was downregulated on HMECs-1 after incubation with oxLDL, in a concentration which predominates in atherosclerotic plaques. Trogan et al. previously demonstrated that phases of hyperlipidemia severely reduce CCR7 mRNA and the corresponding protein levels in an ApoE$^{-/-}$-mice model. CCR7 increased in the presence of normolipidemia. In their work, CCR7 expression was found to be essential for the migration of DCs from atherosclerotic plaque. In atherosclerotic plaques of ApoE$^{-/-}$-mice, CCR7 receptor expression was reduced. When transferred to wild-type mice, this process was accompanied by an upregulation of CCR7 itself [32].

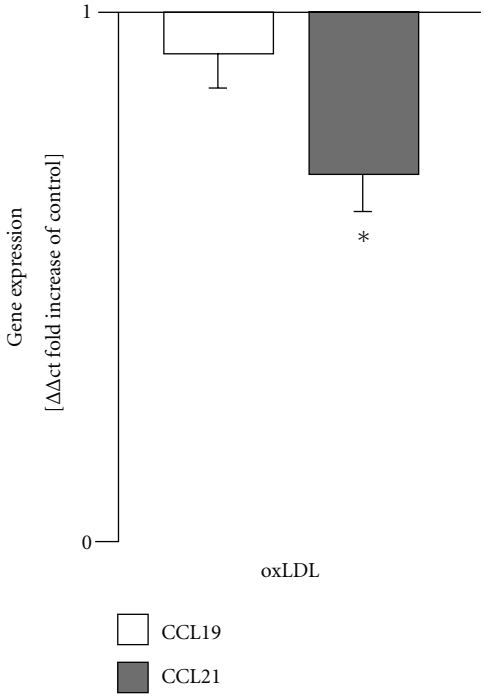
Under a high-dose statin therapy, Damas et al. found a significant decrease in CCL19 and CCL21 levels in serum [2]. Our data support this observation, showing reduced CCL21 serum protein expression in the control group, characterized by low oxLDL levels. The difference in controls was evident despite a low-dose statin therapy.

Our results suggest that DC maturation is triggered by increased oxLDL deposits in the subintimal space. At the same time DCs, and even T-cells do not seem to express enough CCR7 receptors in order to migrate. As a consequence, oxLDL, DCs and T-cells appear to accumulate, thereby enhancing plaque inflammation processes and possibly plaque rupture.

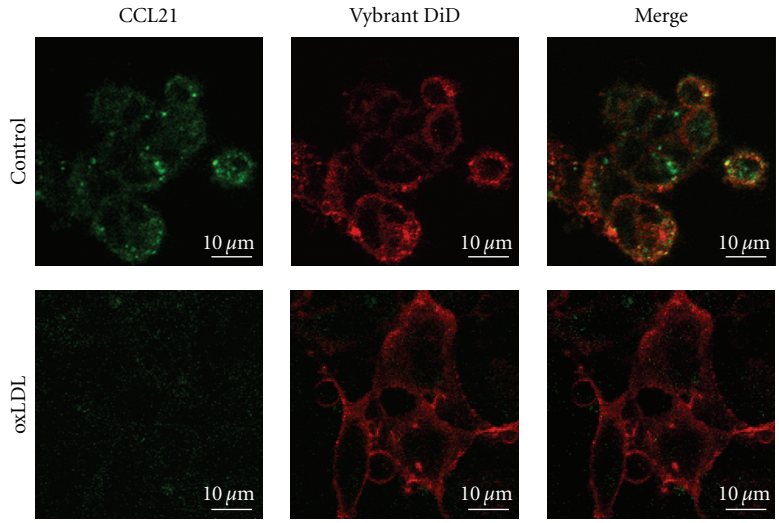
These results complement the former findings that oxLDL increased the adhesion and promotes the maturation and differentiation of DCs from monocytes [9, 26]. Furthermore, oxLDL supports the building of foam-cell accumulations and also directly induces chemotaxis of immune cells like T-cells via upregulation of endothelial adhesion molecules [9]. Keeping in mind that DCs are responsible for priming naive T-cells to oxLDL-specific T-cells [9, 33], the interaction of oxLDL and DCs must be taken into account as a key factor for the induction and progression of atherosclerosis.

oxLDL not only seems to have an influence on the expression of CCR7, but also to induce downregulation of CCL21 on ECs *in vitro* as well as decreased expression of CCL21 and CCL19 in the plaque. Comparing CCL19 with CCL21, the latter appears to be affected more directly by local plaque homing factors. In plaque versus normal aortic tissue, we found a much higher response to CCL21 on ECs as opposed to CCL19. Typically, the expression of CCL21 on ECs is higher than CCL19 [11], since CCL21 has a C-terminus indicating a strong affinity to glycosaminoglycan. This is crucial for effective presentation of CCL21 on ECs [11, 34].

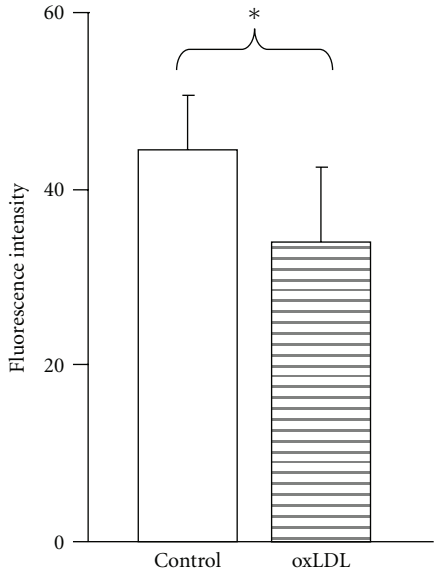
In contrast to our study, other investigators demonstrated an upregulation of CCL21/CCL19 in carotid plaques [7]. In previous studies, 2–4 coronary or renal vessels from autopsy material were taken as controls. To overcome these limitations, we used 14 age-matched healthy aortic tissue samples taken from valve operations serving as controls, which were immediately frozen after their operative preparation, similar to the procedures of carotid tissue preparation. Autolysis and coincidence caused by the small number of controls were therefore ruled out in our experimental setting. A further aspect which points against the renal vessels as controls is the fact, that the vascular-associated lymphoid tissue (including DC and T-cells) has been characterized in human large arteries, including the aorta, carotid, and coronary arteries [35, 36] but not in renal vessels.



(a) mRNA expression of CCL21



(b) Protein expression of CCL21



(c) Quantification of the fluorescence intensity

Figure 4: mRNA expression of CCL21/19 and protein expression of CCL21 on HMECs-1 after stimulation with oxLDL. (a) Fold increase in mRNA expression levels of CCL21/19 in human microvascular endothelial cells after 48 h of stimulation with oxLDL (10 μg/mL) analyzed by real-time PCR. $^*P < 0.05$, ($n = 9$) versus unstimulated controls. (b) Corresponding protein expression of CCL21 measured by immunofluorescence analysis. The green staining represents CCL21 positive structures (α-CCL21/6Ckine-Alexa488), red represents the plasma membrane (Vybrand DiD). Scale bar 10 μm. (c) The fluorescence intensity was quantified showing a significant change for oxLDL. $^*P < 0.05$, ($n = 8$) versus unstimulated control.

5. Conclusions

In summary, our results suggest that alterations in the vascular chemokine profile may be responsible for accumulation of mature DCs in plaque, which potentially enhance the risk for plaque rupture. It may be argued that inhibiting DC migration leads to a less robust immune response. Indeed, altered priming can be expected in the presence of impaired DC migration. However, if migration is blocked and maturation enabled, the consequence of trapping mature DCs in the direct atherogenic vicinity may induce plaque formation and progression or even rupture. Suppression of DC migration and maturation may, therefore, prove a promising future therapeutic target to prevent plaque instability and rupture.

Acknowledgments

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Research Article

Pregnancy Followed by Delivery May Affect Circulating Soluble Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Levels in Women of Reproductive Age

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Background/Objective. It is known that menopause or lack of endogenous estrogen is a risk factor for endothelial dysfunction and CAD. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is involved inmultiple phases of vascular dysfunction.The purpose of the current study was to determine the association between soluble LOX-1 (sLOX-1) and pregnancy followed by delivery in women of reproductive age. *Materials/Methods*. Sixty-eight subjects with pregnancy followed by delivery (group 1) and 57 subjects with nongravidity (group 2) were included in this study. Levels of sLOX-1 were measured in serum by EL SA. *Results*. Plasma levels of sLOX-1 were significantly lower in Group 1 than Group 2 in women of reproductive age (0.52 ± 0.18 ng/mL and 0.78 ± 0.13, resp., $P < 0.001$). There were strong correlations between sLOX-1 levels and the number of gravida ($r = -0.645$, $P < 0.001$). The levels of sLOX-1 highly correlated with the number of parous ($r = -0.683$, $P < 0.001$). *Conclusion*. Our study demonstrated that serum sLOX-1 levels were associated with pregnancy followed by delivery that might predict endothelial dysfunction. We conclude that pregnancy followed by delivery may delay the beginning and progress of arteriosclerosis and its clinical manifestations in women of reproductive age.

1. Introduction

Even though substantial efforts have been made to improve education and public awareness and despite the use of effective medications and life-style changes for controlling the associated risk factors, coronary artery disease (CAD) remains the leading cause of death in women worldwide [1, 2]. In contrast to age-matched men, the incidence of clinical manifestations of CAD is considerably lower in premenopausal women; however, most of women develop CAD after menopause when endogenous estrogen levels are low [3–5].

During normal menstrual cycles, women show high levels of estrogen just before ovulation and during the luteal phase and in the normal physiology of pregnancy, women have significantly higher levels of estrogen derived mainly from the placenta [6]. Estrogens have been known to exert various positive effects on the cardiovascular system [7, 8]. It has thus been shown that estrogens retard the atherosclerotic process and induce rapid vasodilatation through the production of an endothelium-derived vasoactive mediator, nitric oxide (NO) [8–10]. Hashimoto et al. [11] reported that endothelium-dependent vasodilatation is increased in young women during the phases of their menstrual cycles when endogenous estrogen levels are high, and pregnant women show significantly high levels of estrogen. Some studies have documented that estrogens are potent antioxidants and decrease low-density lipoprotein cholesterol (LDL-C) oxidation in vitro and in vivo [12, 13]. While estrogens decrease lipid peroxidation and formation of reactive oxygen species [14], androgens and progestins increase oxidative stress parameters [15].

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), a type II membrane glycoprotein, is the major receptor for oxidized low-density lipoprotein (ox-LDL) in endothelial cells [16]. Oxidative stress and ox-LDL both alter

endothelial biology by activating a specific receptor LOX-1. The activation of LOX-1 has been shown to lead to further oxidative stress in endothelial cells and the appearance of proinflammatory phenotype [17]. LOX-1, furthermore, is cleaved at the membrane-proximal extracellular domain by proteases [18, 19] that may also be associated with endothelial dysfunction and atherosclerotic plaque formation and destabilization, resulting in soluble LOX-1 (sLOX-1) release into the circulation [19]. Since the level of soluble receptors in circulating blood may reflect the expression of membrane proteins and disease activities, sLOX-1 may be a potential biomarker of vascular disease assessment.

Therefore, we hypothesized that if women have been exposed for a longer time and/or at a higher level to endogenous (not exogenous) estrogen, such as pregnancy followed by delivery and/or gravidity, they may obtain estrogen's beneficial "cardioprotective," "antiatherosclerosis," and/or "antioxidant" effect. The purpose of the current study was to determine the association between pregnancy followed by delivery and sLOX-1.

2. Methods

2.1. Patient Population. From January 2010 to June 2011, we prospectively evaluated 1284 patients in cardiology outpatient clinic of our hospital. Sixty-eight subjects with pregnancy followed by delivery (Group 1) and 57 subjects with nongravidity (Group 2) were included in this study. All participants provided written informed consent to participate in the study. The protocol was approved by the local Ethics Committee.

Exclusion criteria included pregnancy, known polycystic ovary syndrome, congestive heart failure (ejection fraction <50%), myocardial infarction, stroke, known peripheral atherosclerotic disease, surgical coronary intervention, other major vascular surgical procedures, coronary angioplasty, unstable angina pectoris, diabetes mellitus, hypertension, suspected myocarditis or pericarditis, impaired renal function (creatinine ≥ 1.4 mg/dL), unstable endocrine or metabolic diseases known to influence serum inflammation markers, concomitant inflammatory diseases such as infections and autoimmune disorders, active or chronic hepatic/hepatobiliary disease, and malignancy. Patients taking oral contraceptive, corticosteroids, anti-oxidant vitamins, and alcohol were also excluded from the study.

2.2. Blood Sampling and Laboratory Methods. Blood samples of all individuals were taken from an antecubital vein following an overnight fasting state at the first three days of menarche. After centrifugation at $3000 \times$g for 10 minutes, serum and plasma samples were frozen and stored at -80°C until an assay could be performed. Serum sLOX-1 levels were measured by a commercially available enzyme-linked immunosorbent assay kit (USCN Life Science, Wuhan, China). The detection limit for serum sLOX-1 level was 2.4 pg/mL with a coefficient of variation <5%. Triglyceride (TG), total cholesterol (tot-C), LDL-C, and high-density lipoprotein cholesterol (HDL-C) concentrations were measured by automated chemistry analyzer (Roche Diagnostics, Indianapolis, USA) by using commercially available kits.

3. Statistical Analysis

Continuous variables were given as mean ± SD; categorical variables were defined as percentages. Comparisons between Group-1 and Group-2 were carried out using an independent samples t-test. Correlation analyses were performed using the Pearson coefficient of correlation. SPSS 15.0 software was used for basic statistical analysis (Version 15, SPSS Inc., and Chicago, IL, USA). A value of $P < 0.05$ was accepted as statistically significant.

4. Result

The clinical and demographic characteristics of study subjects were summarized in Table 1. The mean age was 33.5 ± 6.1 years in pregnancy followed by delivery group and 35.5 ± 7.5 years in nongravidity group ($P = 0.1$). The mean age of first menarche was 12.1 ± 2.3 years in pregnancy followed by delivery group and 11.9 ± 1.9 years in nongravidity group ($P = 0.5$). The rates of family history and smoke were similar between the two groups (Table 1). The smoker subjects in Group 1 had 3.5 ± 1.3 pack-year history of smoking, and smoker subjects in Group 2 had 5.0 ± 2.1 pack-year history of smoking ($P = 0.1$). The levels of total-C, LDL-C, HDL-C, and triglyceride were also similar between the two groups (Table 1).

Figure 1 shows the sLOX-1 levels between two groups. The sLOX-1 levels were significantly higher in nongravidity group than pregnancy followed by delivery group (0.78 ± 0.13 ng/mL and 0.52 ± 0.18 ng/mL, resp., $P < 0.001$). The sLOX-1 levels highly negatively correlated with the number of gravida (Figure 2, $r = -0.645$, $P < 0.001$). Figure 3 shows a highly negative correlation between sLOX-1 levels and number of parous ($r = -0.683$, $P < 0.001$). The sLOX-1 levels were not correlated with age and age of first menarche ($r = 0.055$, $P = 0.541$ and $r = -0.015$, $P = 0.865$, resp.). In the multiple linear regression analysis age was positively related and number of gravity was negatively related with sLOX-1 levels (for age $P = 0.011$, beta $= 0.169$, $t = 2,589$ for parous $P < 0.001$, beta $= -0.713$, $t = -10,912$).

5. Discussion

To the best of our knowledge, this is the first study that shows the relationship between sLOX-1 levels and pregnancy followed by delivery in women of reproductive age. This study showed that women who had at least 1 pregnancy followed by delivery showed a decreased level of sLOX-1 compared with those who had never experienced delivery. The sLOX-1 levels had significantly negative correlation with gravida and parous. We hypothesized that if women have been exposed for a longer time and/or at a higher level to endogenous (not exogenous) estrogen, such as pregnancy followed by delivery and/or gravidity, they may obtain estrogen's beneficial effect and may have a greater decrease in level of sLOX-1. These findings may support the idea that

Table 1: The clinical and demographic characteristics of study subjects.

	Group 1 ($n = 68$)	Group 2 ($n = 57$)	P value
Age (years)	33.5 ± 6.1	35.5 ± 7.5	0.1
Age of menarche (years)	12.1 ± 2.3	11.9 ± 1.9	0.5
Smoke (%)	11.8%	15.8%	0.6
Family history (%)	7.4%	5.3%	0.7
Lipid profile (mg/dL)			
Total-C	179 ± 39	183 ± 28	0.4
LDL-C	108 ± 31	113 ± 23	0.3
HDL-C	45 ± 12	45 ± 11	0.7
Triglycerides	120 ± 66	132 ± 58	0.3

Data expressed as mean ± SD or percentage. $P < 0.05$ was accepted as statistically significant. Total-C: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol. Group 1: pregnancy followed by delivery group, Group 2: nongravidity group.

as long as women are exposed to endogenous estrogen they have decreased level of sLOX-1.

Coronary artery disease remains the leading cause of death in the 21st century. Despite the advances in this area, it is still the main cause of death among women in developed countries [20]. The prevalence of CAD in premenopausal women is smaller than in postmenopausal women, when there is an exponential increase, making the risk for women equal to that for men by the age of 65–70 years. This lag concerning the age period at which the frequency of cardiovascular events increases among women as compared to men has been ascribed to the actions of endogenous estrogen on the cardiovascular system, through mechanisms as yet not completely clarified.

The well-known risks for CAD, such as systemic hypertension, smoking, obesity, sedentary life-style, dyslipidemia, stress, family history of CAD, diabetes mellitus, menopause, lack of endogenous estrogen, and insulin resistance, are numerous [21]. More recently, endothelial vascular dysfunction has become suspected as being associated with CAD. The term endothelial dysfunction is more frequently used to refering reduction in endothelium-dependent vasodilatation, associated with diminished bioactivity of local vasodilative factors (especially NO). Data from prospective trials have been confirming the hypothesis that endothelial dysfunction precedes the emergence of chronic disorders. Currently, it is a consensus that endothelial dysfunction is the initial event in development of atherosclerosis [22]. There are many techniques for investigating the endothelial function, from those that focus on cellular and molecular aspects, through methods involving tissue culture and molecular biology tools, to clinical trials applied to human beings, using invasive and noninvasive procedures to evaluate endothelium-dependent vasodilatation, or the determination of plasmatic substances that indicate endothelial activation and/or damage.

The incidence of CAD and mortality is very low in women of reproductive age but rises to a significant level in menopause women [23]. There is evidence of an association between endothelial dysfunction and reduced endogenous production of estrogens after natural or surgical menopause or premature ovarian failure in women with or without CAD [24–27]. The actions of endogenous estrogens on the cardiovascular system can be mediated directly on the vessels or indirectly through the modulation of cardiovascular risk factors, as well as on the lipid profile [28]. The direct effects of estrogen on the vascular system and which modulate the vascular tonus comprise the following 1 acute vasodilatation, increasing the synthesis and bioactivity of NO [29, 30]; 2 long-term modulation of vascular tonus, regulating the production of prostaglandins and expression of endothelial nitric oxide synthase and the endothelin gene [31]; 3 inhibition of endothelin-induced vasoconstriction [32]; and 4 inhibition of sympathetic activity [24]. In addition to these actions on the vascular tonus, estrogen exerts an antiproliferative action on the vascular smooth layer [33]. Also, it appears to have a major role in vascular remodeling, inhibiting the proliferation of the inner layer after injury [34] and increasing the expression of contractile proteins in the myocardium [35].

Disturbances in endothelial function have an important role in the physiopathology of atherosclerosis, and several lines of evidence suggest that interventions in endothelial function could modify the progress rates of atherosclerotic disease and the risk of cardiovascular events. Some studies have documented that estrogens are potent antioxidants and decrease LDL-C oxidation in vitro and in vivo [12, 13]. Studies on the mechanism of estrogen antioxidant effects have shown that estrogen strongly inhibits superoxide formation with minor effects on hydrogen peroxide and hydroxyl radical formation [14]. While estrogen decreases lipid peroxidation and formation of reactive oxygen species, [14] androgens and progestins increase oxidative stress parameters [15]. Clinical studies on humans using 17β-estradiol-based preparations have clearly shown decreased LDL-C oxidation, and in addition, estradiol reduces the development of early lesions of atherosclerosis, in part through the effects on lipid metabolism which reduce lipid deposits in the endothelium [36, 37].

Cevic et al. demonstrated that a high concentration of estrogen reduces the level of asymmetric dimethylarginine (ADMA), which is an endogenous competitive inhibitor of NO synthase [38]. Estradiol, by reducing ADMA, may therefore facilitate NO synthesis in endothelial cells. Hashimoto et al. [39] also demonstrated that women who had had at

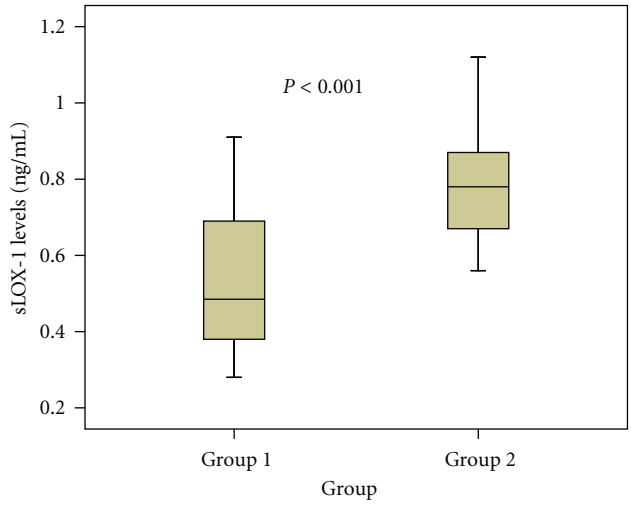

Figure 1: The comparison of sLOX-1 levels between two groups (Group 1: pregnancy followed by delivery group, Group 2: nongravidity group. Soluble LOX-1 levels were 0.78 ± 0.13 ng/mL in nongravidity group and 0.52 ± 0.18 ng/mL in pregnancy followed by delivery group, $P < 0.001$).

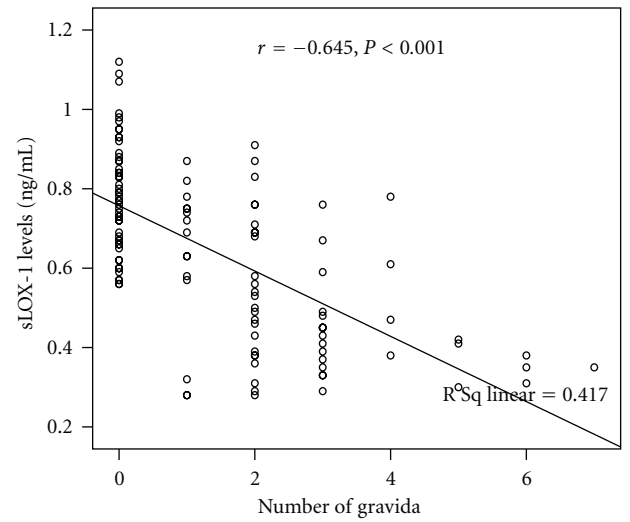

Figure 2: The correlation between sLOX-1 levels and number of gravida.

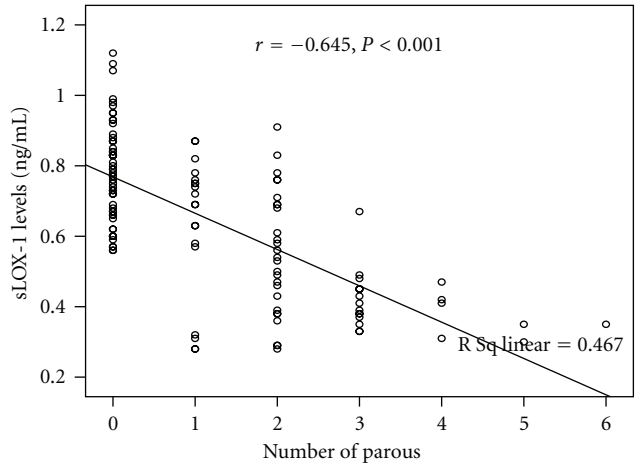

Figure 3: The correlation between sLOX-1 levels and number of parous.

least 1 pregnancy followed by delivery showed a decreased level of arteriosclerosis, measured noninvasively by brachial-ankle pulse wave velocity (ba-PWV) as an indicator of arteriosclerosis. It was closely correlated with aortic arterial stiffness and the severity of atherosclerosis, compared with those who had never experienced delivery. Human umbilical vein endothelial cells exposed to high concentration of 17β-estradiol were used as an antiatherosclerogenic agent to demonstrate feasibility in an in vitro vascular model [40].

LOX-1, a type II membrane glycoprotein, is the major receptor for ox-LDL in endothelial cells [16]. It is also expressed by macrophages and vascular smooth muscle cells [41]. Oxidative stress and ox-LDL both alter endothelial biology by activating a specific receptor LOX-1. The activation of LOX-1 has been shown to lead to further oxidative stress in endothelial cells and the appearance of proinflammatory phenotype [17]. LOX-1 has been implicated in vascular inflammation and atherosclerotic plaque formation, progression, and destabilization [42, 43]. LOX-1, furthermore, is cleaved at the membrane-proximal extracellular domain by proteases, including a disintegrin and matrix metalloproteinases (MMPs) [18, 19] that may also be associated with plaque vulnerability or rupture, resulting in soluble LOX-1 (sLOX-1) release into the circulation [19]. In addition, plasma sLOX-1 levels were higher in males and smokers than in females and nonsmokers, probably because endogenous estrogen and smoking affect plaque vulnerability by protecting vascular cells from inflammation (the former) [8] and by inducing oxidative stress and inflammation (the latter) [44]. In experimental animal models, LOX-1 expression is closely associated with morphological plaque instability and cell apoptosis, as well as with the expression of MMPs and tissue factor, all of which are associated with plaque rupture and thrombus formation [45–47]. A study demonstrated that LOX-1 deficiency significantly decreases the formation of atherosclerotic lesions and endothelial dysfunction [48].

It is well known that menopause or lack of endogenous estrogen is a risk factor for cardiovascular disease [49–51]. Hashimoto et al. [39] reported that women who are regularly menstruating have a decreased PWV compared with postmenopausal women of the same age and a younger age at menarche correlates with PWV reduction. This finding may support the idea that as long as women are exposed to endogenous estrogen they have decreased endothelial dysfunction. Cardiovascular risk increases after bilateral ovariectomy and in conditions associated with impaired ovarian function. Thus, ovarian dysfunction and either natural or surgical menopause have been recognized as a major risk factor for accelerated atherosclerotic vascular disease development [3, 52]. In stages of disrupted ovulatory cycling,

low levels of endogenous oestrogens during premenopausal years accelerate the progression of atherosclerosis [53, 54], which can be reversed by oestrogen therapy in animals [53]. In addition, results from experimental studies and recent clinical trials indicate that oestrogen therapy started within few years after menopause, that is, before the development of severe atherosclerosis, may in fact reduce cardiovascular risk [5, 55–59]. In contrast, initiation of oestrogen therapy many years after menopause, that is, when advanced and multiple atherosclerotic lesions are present, may have no or even deleterious cardiovascular effects [5, 55–59].

In conclusion, our study demonstrated that serum sLOX-1 levels were associated with pregnancy followed by delivery which might predict endothelial dysfunction. Pregnancy followed by delivery may improve endothelial function and prevent the progress of atherosclerosis in women of reproductive age. LOX-1 can be used as a target for imaging of endothelial function. We conclude that pregnancy followed by delivery may delay the progress of arteriosclerosis and its clinical manifestations in women of reproductive age.

Conflict of Interests

The authors declare that they have no conflict of interests.

Author's Contributions

M. Balin and A. Celik conceived and designed the study. M. A. Kobat and A. Baydas assembled the data. A. Celik analyzed and interpreted the data. M. Balin, A. Celik, M. A. Kobat and A. Baydas provided materials and analysis tools. M. Balin and A. Celik jointly wrote the paper. All authors participated in the preparation of the manuscript and gave their final approval of the paper.

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Review Article

Role of Leukotrienes on Protozoan and Helminth Infections

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Leukotrienes (LTs), formed by the 5-lipoxygenase-(5-LO-) catalyzed oxidation of arachidonic acid, are lipid mediators that have potent proinflammatory activities. Pharmacologic or genetic inhibition of 5-LO biosynthesis in animals is associated with increased mortality and impaired clearance of bacteria, fungi, and parasites. LTs play a role in the control of helminth and protozoan infections by modulating the immune system and/or through direct cytotoxicity to parasites; however, LTs may also be associated with pathogenesis, such as in cerebral malaria and schistosomal granuloma. Interestingly, some proteins from the saliva of insect vectors that transmit protozoans and secreted protein from helminth could bind LTs and may consequently modulate the course of infection or pathogenesis. In addition, the decreased production of LTs in immunocompromised individuals might modulate the pathophysiology of helminth and protozoan infections. Herein, in this paper, we showed the immunomodulatory and pathogenic roles of LTs during the helminth and protozoan infections.

1. Introduction

Leukotrienes (LTs), first described by Samuelsson's group [1, 2], are a class of lipid mediators involved in several diseases but classically known for their effects on asthma and allergy. The generation of leukotrienes (LTs) is dependent upon the action of 5-lipoxygenase (5-LO) in association with membrane-bound 5-lipoxygenase-activating protein (FLAP) on arachidonic acid (AA). AA is derived through the action of cytosolic phospholipase A_2 ($cPLA_2$) and/or secreted phospholipase A_2 ($sPLA_2$) on membrane phospholipids [3]. LTA_4, an unstable precursor of all leukotrienes, is quickly metabolized to one of the two different classes of LTs, LTB_4 (by LTA_4 hydrolase) or LTC_4 (by LTC_4 synthase) and its metabolites (LTD_4 and LTE_4) [4]. Collectively, LTC_4, LTD_4, and LTE_4 were previously known as the slow-reacting substance of anaphylaxis (SR-A) and are currently termed the cysteinyl LTs (cysLTs) [3, 4]. The receptors for LTB_4 (BTL1 and BTL2) and cysteinyl LTs (CysLT1 and CysLT2) are cell surface G protein-coupled receptors [3]. Additionally, some studies support the existence of other CysLT receptors [5, 6]. Some cells express both BTLs and cysLTs; however, the expression of these receptors differs in different cells types. In addition, these receptors are also expressed on peripheral blood leukocytes [7, 8]. LT receptors and 5-LO are expressed mainly in immune cells [6], and LTs play important roles in innate and adaptive immune responses and are involved in several inflammatory and infectious diseases [4, 9]. For example, cysLTs increase vascular permeability and edema, and LTB_4 is involved in leukocyte chemotaxis, lysosomal enzyme secretion, neutrophil degranulation, adhesion molecule expression, defensins and nitric oxide (NO) production, phagocytosis, and other functions [9]. LTs are produced during the interaction of phagocytes and microorganisms *in vitro* and experimental infections *in vivo* [9]. Pharmacologic or genetic approaches to reduce or block the LT biosynthesis pathways decrease the phagocytic and antimicrobial activities against bacteria [10], fungi [11], and parasites [12, 13]. In addition, immunodeficient individuals, such as HIV patients, are characterized by low LT production [14], which has been associated with impaired immune responses and infection control. LTs play important roles in both Th1 and Th2 immune responses, which are involved in the defense against protozoan and helminth infections,

respectively. In light of the current research on the role of LTs in infectious diseases, we have divided the current review into two sections focusing on (1) protozoan infection and (2) helminth infection.

2. Leukotrienes and Protozoan Infection

Each year, protozoan parasites infect many people worldwide, mainly in developing countries, causing serious health, political, social, and economic problems. The major protozoan parasites with clinical importance for human diseases are *Plasmodium* ssp, *Leishmania* ssp, *Trypanosoma cruzi*, *Toxoplasma gondii*, *Trichomonas vaginalis,* and *Entamoeba histolytica* [15–17]. The first three of these organisms are obligate intracellular protozoan parasites that are transmitted to vertebrate hosts by insect vectors. *T. gondii* is also an obligate intracellular protozoan parasite; however, its transmission to human hosts occurs by ingestion of raw or undercooked meat containing tissue cysts or food or water contaminated with oocysts. *T. vaginalis* and *E. histolytica* are extracellular protozoan parasites. *T. vaginalis is* transmitted sexually (trophozoites) and *E. histolytica* is transmitted through food and water contaminated with cysts [15–17]. Protective immunity against protozoans is mediated mainly by T helper 1 (Th1) responses which are characterized by the production of inflammatory cytokines, such as IL-12, which is required for the development of the Th1 immune response, and interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), which activate macrophages to produce NO, which is involved in the control of parasite replication [16, 18–20].

Reiner and Malemud [21, 22] conducted the first studies to demonstrate the role of leukotrienes in protozoan infection (*Leishmania* spp). Research in this area has increased in recent decades. The main effects of LTs, in both innate and adaptative immune responses, during the protozoan infections are illustrated in Figure 1. Mouse strains resistant (C57BL/6) to *Leishmania* infection mount Th1 immune responses against *Leishmania*. In contrast, infection of susceptible mouse strain (BALB/c) is associated with the development of a Th2 immune response. *In vitro* studies have demonstrated increased LTC_4 production in splenocytes and macrophages from *L. donovani*-infected or uninfected BALB/c mice upon stimulation with nonspecific (phytohemagglutinin) or specific (*L. donovani* amastigotes) stimuli [21, 22]. In another study, splenocytes from BALB/c mice stimulated with antigens from *L. major* promastigotes displayed increased LTB_4 and IL-4 production with concomitant decreases in IFN-γ and TNF-α production [23]. Serezani et al. [24] demonstrated an increase in the parasite burden of BALB/c macrophages infected with *L. amazonensis* when compared to macrophages from the resistant mouse strain C3H/HePas. This effect was associated with lower levels of LTB_4 in macrophages from BALB/c mice. In agreement with this finding, macrophages from either susceptible or resistant mice treated with MK0591 (FLAP inhibitor) and U75302 (BLT1 antagonist), but with not MK571 (cysLT1 antagonist), as well as macrophages derived from 5-LO-deficient mice, exhibited decreased leishmanicidal activity. Interestingly, treatment with exogenous LTB_4 or LTD_4 favored parasite killing by macrophages from BALB/c mice. Supporting these *in vitro* results, susceptible and resistant mice treated with zileuton (inhibitor of 5-LO) or 5-LO-deficient mice infected with *L. amazonensis* displayed larger footpad lesions than nontreated or wild type animals [24].

The success of *Lutzomyia longipalpis*, an insect vector of the *Leishmania* ssp, at blood feeding on mammals depends on the inhibition of the immediate inflammatory response (e.g., increased vascular permeability, swelling, pain, and itching). It is well known that active substances in the saliva of hematophagous arthropods facilitate the uptake of blood by counteracting host hemostatic, inflammatory and immunological defenses [25–28]. Mixed lysates from the salivary glands of *L. longipalpis* significantly increased the cutaneous lesions and/or parasite loads in the footpads of mice infected with *L. major* or *L. braziliensis* when compared to infected animals not exposed to the saliva lysates [29, 30]. In addition, the modulation of infection by saliva was IL-4-dependent [29]. In agreement with these results, the salivary gland extract of *L. longipalpis* exhibited anti-inflammatory activities by decreasing TNF-α and LTB_4 production, neutrophil numbers, and LTB_4-induced chemotactic activity in a murine ovalbumin-induced peritonitis model [31]. In addition, IL-10 and IL-4 production was increased in this model. Taken together, these findings suggest that LTs, and particularly LTB_4, play a role in immune response to *Leishmania* infection by promoting leishmanicidal activity and consequently, control of infection. Therefore, the modulation of LTB_4 during infection in association with the modulation of the immune system during *Leishmania* transmission (by saliva from the insect vector) in synergism with genetic factors (susceptibility; Th2) could markedly affect *Leishmania* infection in humans.

The components derived from the saliva of the arthropod vector of malaria (e.g., *Anopheles stephensi*) have also pharmacologic effects, such as inhibition of inflammation and coagulation [32], similar to those observed in the saliva of insect vectors of *Leishmania*. In addition, these proteins also have the ability to neutralize inflammatory small molecules by rapid binding. The AnSt-D7L1 protein produced by *A. stephensi* binds cysLTs (LTC_4, LTD_4, and LTE_4) but does not chemically modify them. AnSt-D7L1 effectively inhibited LTC_4-induced ileal contraction by binding LTC_4, thereby preventing interactions between this molecule and its appropriate cellular receptor [25]. The effects of LTC_4 inhibition on the course of malaria infection as well as the influence in the malaria pathogenesis are not known.

In the experimental cerebral malaria model, mice infected with *Plasmodium berghei* showed increased LTB_4 production in the serum. Interestingly, treatment with aspirin, which may direct arachidonic acid metabolism away from the cyclooxygenase (COX) pathway and toward the LO pathway [33], induced increased parasitemia and death of infected mice. This effect was associated with the overproduction of LTB_4 in the serum [34]. In agreement with these results, children with cerebral malaria treated with salicylate demonstrated complications of severe malaria (metabolic

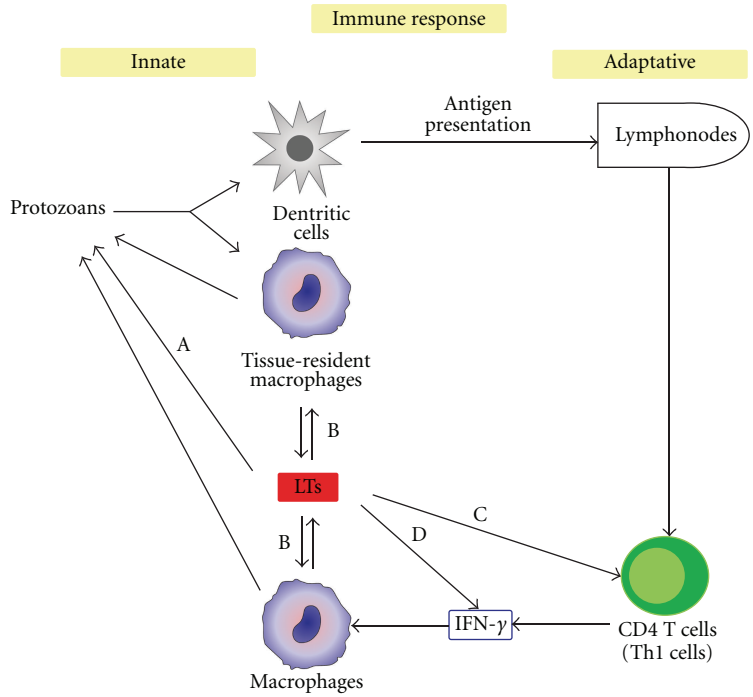

Figure 1: Modulation of innate and adaptive immune responses by LTs during the protozoan infections. Protective immunity against protozoans is mainly mediated by Th1 responses characterized by the production of inflammatory cytokines, such as IFN-γ, which activate macrophages to control of parasite replication. Macrophages are a source of LTs. (A) LTB_4 induces cytotoxicity in parasites. (B) LTB_4 and LTD_4 favored parasite killing by macrophages. (C) LTB_4 induces chemotaxis of $CD4^+$ T cells. (D) LTB_4 induces the production of Th1 cytokines, such as IFN-γ.

acidosis, hypoglycemia, and death) [35]. Although IFN-γ plays a protective role in malaria infection, it has also been associated with the immunopathology of cerebral malaria [36, 37]. Besides playing a role in initiating the Th1 immune response mediated by dendritic cells [38], LTB_4 is also an inducer of Th1 cytokines, such as IFN-γ [39]. Therefore, the overproduction of LTB_4 after aspirin treatment in experimental and human cerebral malaria could be associated with the overproduction of IFN-γ. Further studies are needed to evaluate this hypothesis.

Eryptosis, or suicidal death of erythrocytes, which occurs in a wide variety of diseases including malaria [40], is characterized by cell shrinkage, membrane blebbing, and exposure of phosphatidylserine (PS) at the cell surface [41]. Like apoptotic cells, PS-exposing erythrocytes are identified by macrophages and are engulfed, degraded, and removed from the circulation [42]. Ayi et al. [43] demonstrated increased phagocytosis of mutant red blood cells infected with trophozoites of *P. falciparum*, which may represent a protective mechanism against infection. Remarkably, an *in vitro* assay demonstrated that erythrocytes were able to produce cysLTs upon energy depletion [44]. In addition, exogenous treatment with LTC_4, but not LTB_4, stimulated eryptosis. These effects were inhibited by cysLT1 receptor antagonists and by the 5-LO inhibitor (BW B70C) [44]. These results suggest that LTC_4 might confer protection during the course of malaria by accelerating the clearance of infected erythrocytes. On the other hand, excessive eryptosis might favor the development of anemia; thus, LTC_4 might have a dual effect in malaria pathogenesis.

During *T. gondii* infection, an efficient immune response is important to contain dissemination of the parasite and to prevent mortality of the host. LTC_4, LTD_4, and free AA were detected when murine macrophages from Swiss mice were cultured with viable *T. gondii* [45]. In contrast, when macrophages from resistant mice (BALB/c; major histocompatibility complex haplotypes $H2^d$) [46] or human macrophages [47] were cultured with viable *T. gondii*, no 5-LO products were observed. Accordingly, prior incubation of human macrophages with viable *T. gondii* decreased the LTB_4 release induced by the calcium ionophore A23187, suggesting that *T. gondii* inhibits LTB_4 production. This effect was restored by IFN-γ treatment [47]. In addition, treatment with zileuton (an inhibitor of 5-LO) decreased the toxoplasmacidal activity of IFN-γ in human macrophages, whereas exogenous LTB_4 promoted intracellular killing of ingested *T. gondii* in human monocytes [47]. This effect might be associated with the effect of LTB_4 on the induction of cytotoxicity (surface membrane vesiculation, extravasation of cytoplasmic contents into a space between the intermembrane spaces and cytoplasmic vacuolization) in *T. gondii* tachyzoites [47, 48]. In agreement with these results, 5-LO-deficient mice infected with *T. gondii* displayed decreased survival as a consequence of an excessive inflammatory response characterized by elevated IL-12 and IFN-γ concentrations in the serum and $CD4^+$ and $CD8^+$ T-cell infiltration in the brain tissue and not of increased parasitic burden [49]. The increased inflammation in the absent of LTs might indicate a compensatory mechanism to control the parasite infection. Taken together, these findings suggest

that the downregulation of LTs production, and particularly of LTB_4, by *T. gondii* might be considered an evasion mechanism, as this lipid mediator can promote cytotoxicity and toxoplasmacidal activity. Thus, LTB_4 plays an important role in toxoplasmosis.

Studies by our group and others have demonstrated reduced LT synthesis (e.g., LTB_4) in HIV-infected subjects [14, 50]. Although the clinical manifestation of *T. gondii* infection is usually asymptomatic in immunocompetent individuals, immunocompromised individuals, such as HIV-seropositive patients, exhibit reactivation of latent tissue cysts (bradyzoites become tachyzoites) and consequent toxoplasmic encephalitis or retinochoroiditis [51, 52]. Interestingly, in agreement with these results, the LTB_4 and LTC_4 concentrations in the cerebrospinal fluid of HIV-1-seropositive patients with toxoplasmic encephalitis but not those of HIV-1-seropositive patients without inflammatory disease or encephalitis were below the detection limit [53]. These results support those described above and suggest that the reduced basal production of LTs in HIV-1-seropositive patients synergizes with the suppression of LTs by *T. gondii*. Moreover, this synergistic decrease in LT production might contribute to the pathogenesis of cerebral toxoplasmosis through the increased reactivation of bradyzoites from tissue cysts and the reduced control of the parasitic infection.

Protective immunity against toxoplasmosis and Chagas disease is mediated by Th1 cells, $CD8^+$ T cells, and IFN-γ [16]. Chagas' heart disease is a severe clinical manifestation of *Trypanosoma cruzi* infection [54]. In chronic Chagas disease, cardiomyopathy is observed as an inflammatory process characterized by the infiltration of T cells and macrophages, resulting in myocarditis, fibrosis, and heart fiber damage [54]. Treatment with LT inhibitors has demonstrated beneficial effects in cardiovascular pathologies [55, 56]. T lymphocytes from patients with chronic Chagas' heart disease [57] or from chagasic mice [58] show increased contractile activity (positive inotropic and chronotropic effects) of heart (atrial) in an *in vitro* assay. Interestingly, pretreatment with lipoxygenase inhibitors (NDGA) or a cysLT receptor antagonist (FPL 55712) decreased this effect. In a separate study, LTC_4 production was observed in the supernatants of murine atria cocultured with T lymphocytes from chagasic mice [58]. In accordance with these results, LTB_4 induces chemotaxis of lymphocytes ($CD4^+$/$CD8^+$ T cells) [8, 59]. Therefore, LTs might modulate the cardiac pathology of Chagas disease by modulating the immune response profile during this infection.

LTB_4 [60] and LTC_4 [61] also increased the phagocytic and trypanocidal activity of murine macrophages incubated with *T. cruzi* trypomastigotes *in vitro*. In addition, LTB_4 restored NO and TNF-α levels, which were decreased by an LTB_4 receptor antagonist (CP-105,696) [62]. CP-105,696 treatment also decreased the trypanocidal activity of IFN-γ in murine macrophages. With the use of pharmacologic (LTB_4 receptor antagonist and LO inhibitors) and genetic approaches (5-LO-deficient mice), researchers have demonstrated increased parasitemia in mice infected with *T. cruzi* [63–65]. In addition, the following anti-inflammatory profiles were observed in *T. cruzi* infection: (1) decreased leukocyte infiltration in the heart; (2) reduced numbers of $CD4^+$, $CD8^+$, and IFN-γ-producing cells in the heart; (3) decreased fibrosis in cardiac tissues; (4) decreased iNOS expression and NO production in the heart; (5) decreased TNF-α and IFN-γ in the heart; (6) increased IL-10 in the heart; and (7) decreased oxidative stress in erythrocytes [63–65]. The survival of 5-LO-deficient mice was greatest when the animals were infected with low number of parasites [64] when compared to animals infected with higher number of parasites [65]. Taken together, these findings suggest that LTs, and specifically LTB_4, play important roles in the control of Chagas disease.

Trichomoniasis is the most common sexually transmitted disease. The supernatant of viable *T. vaginalis* induced increased LTB_4 production in neutrophils in an IgG- and complement-(C5-) dependent manner. This effect was decreased by SC-41930 (LTB_4 antagonist) treatment [66]. In the vaginal discharges from patients with vaginal trichomoniasis, Shaio and Lin [67] demonstrated a positive correlation between neutrophils and LTB_4 production in symptomatic patients when compared to asymptomatic patients. These results suggest that LTB_4 is involved in the inflammation and symptoms of trichomoniasis. The most relevant effects of LTB_4 in protozoan infections are illustrated in Figure 2.

Entamoebiasis causes high morbidity and mortality in the developing world. Peritoneal and splenic macrophages from naïve mice incubated directly with *E. histolytica* trophozoites or with their excretory/secretory products show increased LTC_4 production. On the other hand, peritoneal and splenic macrophages from *E. histolytica*-infected mice produced low levels of LTC_4. Interestingly, amoebic liver abscess-derived macrophages were unable to produce LTC_4 [68]. The downregulation of LTC_4 by *E. histolytica* in inflammatory but not naïve macrophages might be associated with the pathogen's evasion mechanisms.

3. Leukotrienes and Helminthic Infections

Over one-third of the human population is infected with one or more species of helminths [69, 70]. Although host immune responses attempt to control or expel the parasites, these organisms can develop evasion strategies to modulate the innate and adaptive immune responses, allowing them to survive. The most prevalent human helminthiases are caused by nematodes (e.g., *Ascaris lumbricoides*, *Strongyloides* spp., *Enterobius vermicularis,* and *Trichuris trichiura*), including filarial worms (e.g., *Brugia malayi* and *Wuchereria bancrofti*), hookworms (e.g., *Ancylostoma duodenale* and *Necator americanus*), and trematodes (*Schistosoma* spp).

Asthma and helminthiasis present similar features and are both controlled by a $CD4^+$ T-cell immune response. Initial exposure of the immune system to allergic or parasitic antigens leads to the activation of a subset of T cells known as Th2 cells, which orchestrate the immune response to these exogenous antigens by secreting cytokines, including IL-4, IL-5, and IL-13 [71–74]. In addition, the accumulation of eosinophils in the blood (eosinophilia), as well as in different

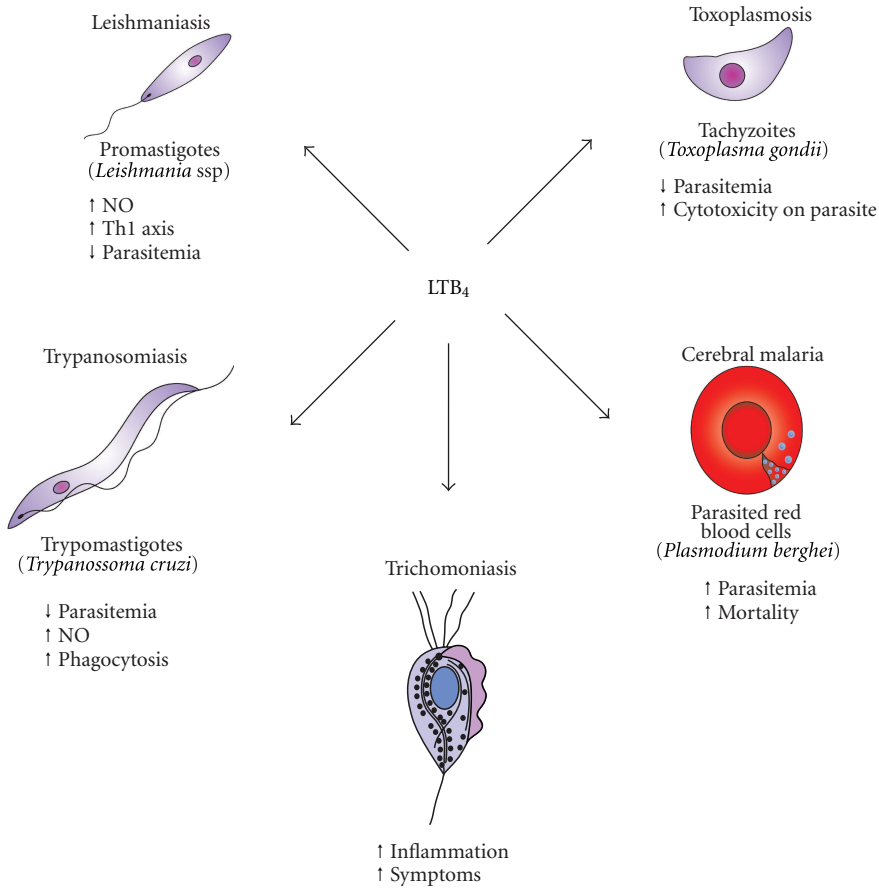

Figure 2: Roles of LTB_4 on protozoan infections. LTB_4 is involved in the control of leishmaniasis, toxoplasmosis, and trypanosomiasis. LTB_4 is also involved in the inflammation and symptoms of trichomoniasis. The overproduction of LTB_4, after aspirin treatment, could be associated with the exacerbated pathogenesis of cerebral malaria.

organs and tissues [75], is a hallmark of both diseases. Eosinophils are multifunctional cells that are involved in tissue damage as a consequence of the release of cationic proteins [76–79]. In addition, eosinophils are important sources of various inflammatory and regulatory cytokines, chemokines, and lipid mediators, such as LTs [78, 80, 81].

During a helminth infection such as a nematode infection, most of the IgE produced binds to mast cells and basophils through their high-affinity IgE Fc receptor (FcεRI) [82, 83]. Subsequent exposure of immune cells to parasitic antigen induces the degranulation of IgE-sensitized mast cells and the release of both preformed and newly generated mediators [82]. These mediators, such as LTs, function alone or in conjunction with Th2 cytokines to increase the contractility of smooth muscle cells, the permeability of epithelial cells and the production of mucus, thereby contributing to worm expulsion [84]. The experimental gastrointestinal infection of rats with the nematode *Trichinella spiralis* demonstrated that preimmune rats (previously infected with *T. spiralis*) expelled the nematode *T. spiralis* more rapidly than nonimmune rats. This effect was associated with the increased production of LTB_4 and LTC_4 in the gut homogenate as well as the release of rat mast cell protease II (RMCPII) in the serum [85, 86]. LTC_4 causes smooth muscle contraction, increases vascular permeability, and stimulates mucus hypersecretion, and LTB_4 recruits and activates inflammatory cells such as eosinophils to favor the expulsion of helminths. Therefore, leukotrienes released from mast cells may effectively participate in protective immune responses resulting in the rapid expulsion of *T. spiralis* and possibly other helminths. The main effects of LTs, in both innate and adaptative immune responses, during the helminth infections are illustrated in Figure 3.

Parasitic worm survival in the host for longer periods depends on the ability of the parasite to evade the host immune system. The ABA-1 protein from *Ascaris lumbricoides* (human parasite) and *Ascaris suum* (pig parasite) is released by larvae and adult organisms [87, 88]. This protein binds a range of fatty acids, including LTs [89]. The interaction between ABA-1 and leukotrienes might be associated with an evasion mechanism; however, further studies are needed to evaluate the ability of this interaction to inhibit the biologic effects of LTs *in vitro* or *in vivo*.

Brugia malayi is a nematode (roundworm) that can cause lymphatic filariasis in humans. The infective larvae (L3) of *Brugia malayi* are transmitted to a vertebrate host by an insect vector and undergo two molts to develop into adult worms and complete the life cycle [90]. Interestingly,

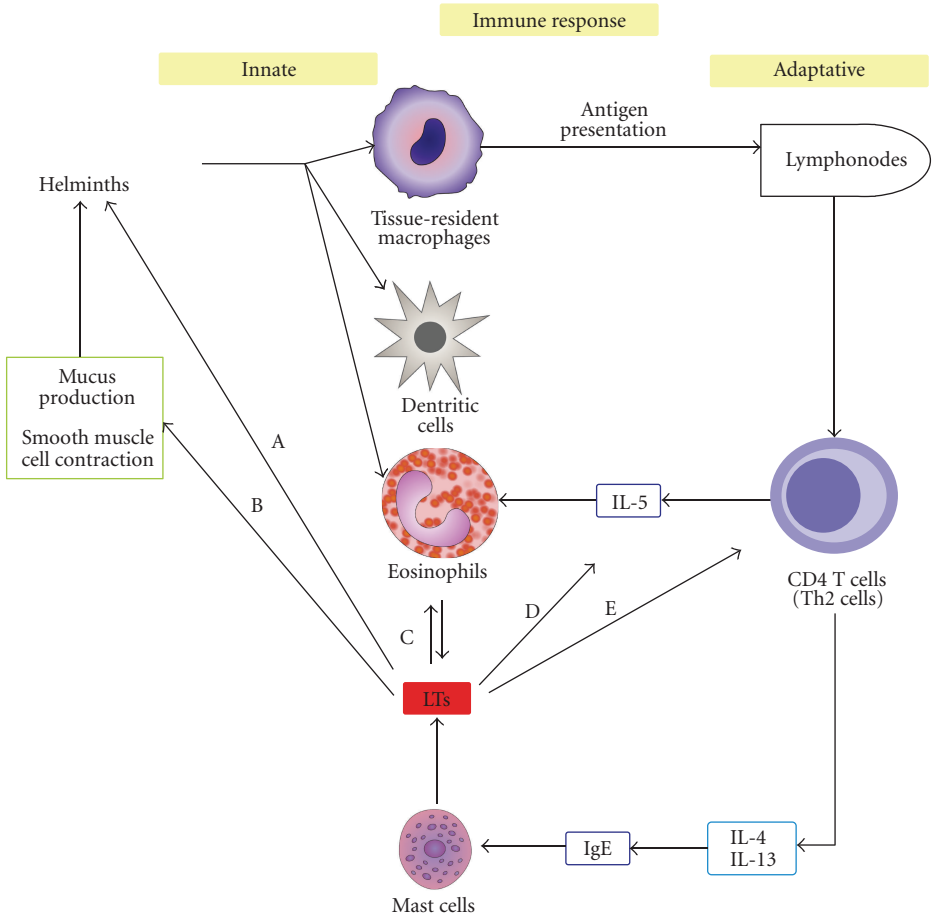

Figure 3: Modulation of innate and adaptive immune responses during the helminth infections. Initial exposure of the immune system to parasitic antigens leads to the activation of a subset of T cells known as Th2 cells, which orchestrate the immune response to these exogenous antigens by secreting cytokines, including IL-4, IL-5 and IL-13. The accumulation of eosinophils in the blood and in different organs and tissues as well as the degranulation of IgE-sensitized mast cells is hallmarks of helminthiasis. Eosinophils and mast cells are sources of LTs. (A) CysLTs are required for molting of the infectious larvae (e.g., *Brugia malayi* larvae). (B) CysLTs, alone or in conjunction with Th2 cytokines, cause contractility of smooth muscle cells, the permeability of epithelial cells, and the production of mucus, thereby contributing to worm expulsion. (C) LTB_4 recruits and activates inflammatory cells such as eosinophils to favor the kill of helminths. (D) LTB_4 regulates IL-5 production by human T lymphocytes and consequently contributes to parasite elimination. (E) LTB_4 induces chemotaxis of $CD4^+$ T cells.

treatment with inhibitors of lipoxygenases (AA861) or cysLT biosynthesis (ethacrynic acid or acivicin) or with a $cysLT_1$ antagonist (zafirlukast) inhibited the *Brugia malayi* L3 larvae from molting to the L4 stage without altering their survival or motility. In contrast, U-75302, an antagonist of the LTB_4 receptor BTL_1, failed to inhibit molting [91]. The γ-glutamyl transpeptidase, the enzyme that converts LTC_4 to LTD_4, has been cloned from *Brugia malayi* (adult worms) [92]. In another filaria that causes human infection, *Dirofilaria immitis*, the glutathione S-transferase, which can function as an LTC_4 synthase, was found in the cytosol of adult worms [93]. These results demonstrated that a lipoxygenase pathway involved in the generation of cysLTs could be required for molting of the infectious larvae and may possibly have some role in the adult worm. *In vivo* models of infection with *B. malayi* could be used to better understand the role of cysLTs in the pathogenesis of filariasis.

It is widely known that some types of infections in immunocompromised individuals are critical in determining the severity of the disease. The immunosuppression observed in HIV-seropositive subjects has been associated with *Strongyloides* spp infections of abnormally high intensity [94]. Interestingly, reduced LT production was observed in HIV-seropositive patients [14]. In an experimental model that mimics human strongyloidiasis (mice infected with *Strongyloides venezuelensis*), an increase in the concentration of LTB_4 but not of LTC_4 was observed in the lung and small intestines. In addition, increased larvae recovery in the lung and/or increased worm burdens in the intestines were observed in animals treated with MK886 (a selective inhibitor of 5-lipoxygenase-activating protein (FLAP)) and in 5-LO-deficient mice than in control animals. Moreover, treatment of animals with MK886 resulted in decreases of IgG_1 and IgE levels in serum, eosinophil numbers in the blood, peritoneal cavity and bronchoalveolar fluid volumes and IL-5 concentrations in the lung homogenate as well as increased levels of IL-12, which is involved in the Th1 response. IL-5 is the major cytokine involved in the accumulation of eosinophils

in the blood during allergic inflammation and parasitic infections. This cytokine is essential for eosinophil migration from the bone marrow to the blood [72, 95] and specifically supports the terminal differentiation and proliferation of eosinophil precursors as well as the activation of mature eosinophils [96–99]. LTB_4 regulates IL-5 production by human T lymphocytes [100] and consequently contributes to parasite elimination. These findings suggest that LTs, and specifically LTB_4, might be necessary to control *S. stercoralis* infection. Thus, the reduced levels of LTB_4 observed in HIV-seropositive subjects might favor opportunistic hyperinfection with *S. stercoralis*; however, further human studies are needed to evaluate this association.

Toxocara canis is an intestinal parasite of dogs and is the etiologic agent of toxocariasis, also known as visceral larva migrans syndrome (VLMS). Infection of both humans and animals with *T. canis* is characterized by eosinophilia in the blood and tissues, increased total serum IgE, and inflammation of the upper respiratory system [72, 95, 101–104]. During the inflammatory response, leukocyte recruitment is directly related to the expression of adhesion molecules, which allows the transmigration of these blood cells to the tissues. The integrin adhesion molecules directly contribute to this process [105]. It has been proposed that the β_2 integrin Mac-1 (CD11b/CD18) and the β_1 integrin VLA-4 (CD49d/CD29) adhesion molecules are the major molecules involved in cytokine- and chemokine-induced adhesion and migration of eosinophils *in vitro* [106, 107]. LTs can enhance the expression of Mac-1 on eosinophil cell surfaces [108]. In mice, *T. canis* infection causes early upregulation of Mac-1 with late changes in VLA-4 profiles on both peritoneal cavity fluid and bronchoalveolar lavage fluids, whereas MK886 treatment promoted the opposite effect. In addition, LT inhibition had a clear impact on eosinophil recruitment to tissues and on blood eosinophilia throughout the course of infection [12]. In another study, in addition to increased eosinophil numbers, the researchers showed increased numbers of mast cells in the peritoneum, lungs, and small intestines of *T. canis*-infected rats. Interestingly, these animals increased the concentration of LTB_4 in the serum and this was correlated with mast cell and eosinophil accumulation and/or recruitment [109]. Thus, LTs might play an important role in eosinophilic inflammation during toxocariasis by inducing leukocytes recruitment and modulating the expression of adhesion molecules.

In schistosomiasis, a granulomatous lesion is observed during chronic infection and causes a range of morbidities [110]. LTs can control parasite infection by modulating immune responses and through direct cytotoxic effects on the parasite. LTB_4, but not cysLTs (LTC_4 and LTD_4), enhanced the ability of neutrophils and eosinophils to kill the schistosomula of *S. mansoni* in a complement-dependent manner [111]. The cytotoxicity of eosinophils against helminths has been associated with the expression of cellular receptors (high affinity IgE receptor, FcεRI) and adhesion molecules and with degranulation and the release of cationic proteins [112]. In an *in vitro* assay, IgE-coated schistosomula induced eosinophil adherence, resulting in the death of the parasites. In addition, the release of LTC_4 was observed during this interaction [113]. In agreement with this finding, schistosomula can produce LTB_4 and LTC_4 [114]. The function of LTs in schistosomula is not known; however, their production might accelerate parasite elimination and/or modulate the pathogenesis of schistosomiasis.

Schistosome cercariae enter mammalian hosts via a percutaneous route [115]. In addition to the proteolytic enzymes produced by cercariae, host-derived skin essential fatty acids and LTs including LTB_4 also play important roles in the penetration of the skin by the parasite. In an *in vitro* assay, increased penetration rates were correlated with increased LTs levels. In addition, penetration was reduced upon treatment with a 5-LO inhibitor [116, 117].

Hepatic granulomatous inflammation is observed during schistosomal infection of both humans and mice [110]. Th2 cell-associated cytokines modulate the development of schistosome egg-induced granulomas. Hepatic stellate cells (HSCs) are involved in liver remodeling due to collagen production and deposition of extracellular matrix as a consequence of proliferative and fibrogenic phenotypes induced by several mediators (cytokines, lipid peroxide, and others) [118]. mRNA for 5-LO, FLAP and LTC_4-synthase and 5-LO expression was observed in HSCs from schistosomal granulomas of *S. mansoni*-infected mice [119]. Consequently, these cells produced cysLTs, but not LTB_4, and the production of cysLTs was increased upon treatment with transforming growth factor beta (TGF-β, a fibrogenic cytokine). The proliferation induced by TGF-β in HSCs from schistosomal granulomas of *S. mansoni*-infected 5-LO-deficient mice or wild type mice treated with zileuton (5-LO inhibitor) was reduced [119]. In addition, LTC_4 induced TGF-β production [120], suggesting a synergic effect in schistosomal granulomas. In another study, dipeptidases were isolated from extracts of hepatic granulomas of mice infected with *S. mansoni*; these enzymes increased the hydrolysis of LTD_4 to LTE_4 [121], potentially accelerating the metabolism of LTs and decreasing their effects on liver remodeling. Moreover, LTB_4 and LTC_4 are produced by schistosomula and adult females, while males produced only LTB_4 [113]. Together, these results suggest that cysLT inhibition might influence liver remodeling in *S. mansoni* infection. In this way, $CysLT_1$ antagonists (such as montelukast, zafirlukast, and pranlukast) [4, 122, 123], which are currently used in asthma treatment, could be evaluated for their effects on schistosomal granuloma remodeling in experimental or human schistosomiasis. The main roles of LTs during the helminth infection are illustrated in Figure 4.

Similar to schistosomiasis, fasciolosis causes liver alterations, which can range from fibrosis to cirrhosis. Fasciolosis is considered both a human health concern and a veterinary problem (zoonoses) [124]. During the course of *F. hepatica* infection in sheep, a reduction in serum LTB_4 was observed when compared to control animals. Interestingly, LTB_4 was produced in both the culture supernatant and the homogenate of *F. hepatica* adult parasites recovered from the bile duct 20 weeks after infection [125]. Moreover, recruitment of leukocytes consisting mainly of eosinophils, macrophages, and lymphocytes was observed in the livers

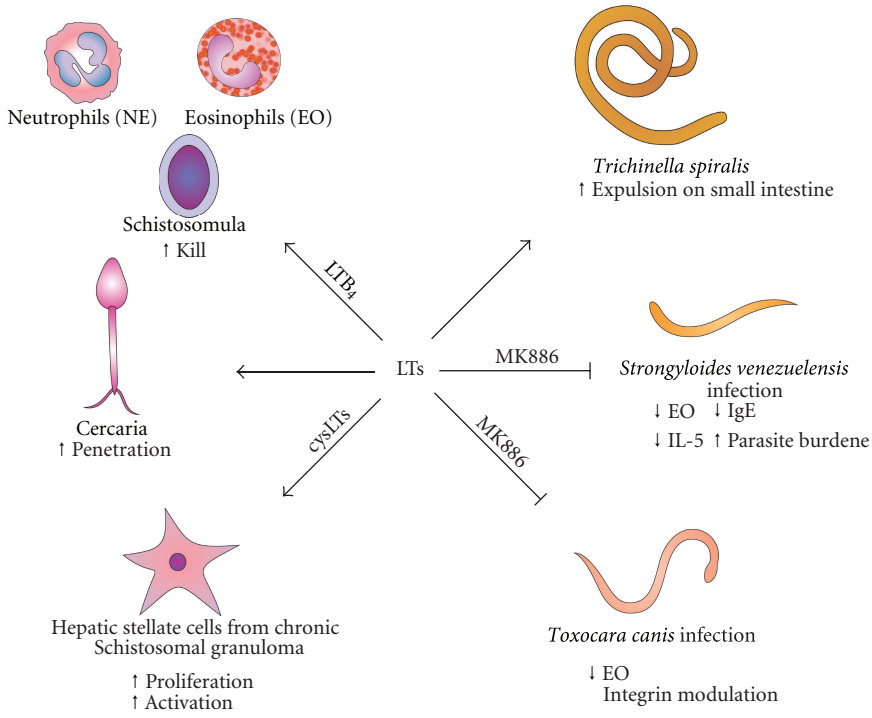

Figure 4: An overview of main effects of LTs in helminth infections. LTs participate of expulsion of *T. spiralis* in the intestine. The inhibition of LTs resulted in increases of parasitemia and decreases IgE levels, eosinophil numbers and IL-5 concentrations in mice infected with *S. venezuelensis*. LT inhibition also reduced the eosinophil recruitment to tissues and on blood eosinophilia in experimental *T. canis* infection. LTB_4 enhanced the ability of neutrophils and eosinophils to kill the schistosomula of *S. mansoni*. LTs play important roles in the penetration of the skin by the schistosome cercariae. CysLTs are involved in the proliferation and activation of hepatic stellate cells from *S. mansoni* granulomas.

of goats infected with *F. hepatica* [126]. In this way, LTB_4 produced by host inflammation in synergy with that produced by the parasite could contribute to liver alterations and consequent pathology.

4. Conclusion

LTs are associated with the control of helminth and protozoan infections through their ability to modulate inflammatory processes and/or to promote direct cytotoxicity of protozoans. In addition, LTs may also be associated with exacerbated pathogenesis in protozoan diseases, such as cerebral malaria, and helminthic diseases, such as schistosomal granulomas. Interestingly, some helminths (*B. malayi*) might use the LTs to complete their development to adult worms. In addition, other parasites produce LTs (*S. mansoni* and *F. hepatica*) or produce enzymes involved in LT biosynthesis (*Dirofilaria immitis*). Taken together, these findings demonstrate that LTs play significant roles in protozoan and helminth infections.

List of Abbreviations

5-LO: 5-Lipoxygenase
AA: Arachidonic acid
cysLTs: Cysteinyl leukotrienes
FLAP: Membrane-bound 5-lipoxygenase-activating protein
HSCs: Hepatic stellate cells
IFN-γ: Interferon-gamma
Ig: Immunoglobulin
IL: Interleukin
LTs: Leukotrienes
NO: Nitric oxide
PS: Phosphatidylserine
TGF-β: Transforming growth factor-beta
Th: T helper
TNF-α: Tumor necrosis factor-alpha
VLMS: Visceral larva migrans syndrome.

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Review Article

Role of PGE_2 in Asthma and Nonasthmatic Eosinophilic Bronchitis

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Eosinophilic bronchitis is a common cause of chronic cough, which like asthma is characterized by sputum eosinophilia, but unlike asthma there is no variable airflow obstruction or airway hyperresponsiveness. Several studies suggest that prostaglandins may play an important role in orchestrating interactions between different cells in several inflammatory diseases such as asthma. PGE_2 is important because of the multiplicity of its effects on immune response in respiratory diseases; however, respiratory system appears to be unique in that PGE_2 has beneficial effects. We described that the difference in airway function observed in patients with eosinophilic bronchitis and asthma could be due to differences in PGE_2 production. PGE_2 present in induced sputum supernatant from NAEB patients decreases BSMC proliferation, probably due to simultaneous stimulation of EP2 and EP4 receptors with inhibitory activity. This protective effect of PGE_2 may not only be the result of a direct action exerted on airway smooth-muscle proliferation but may also be attributable to the other anti-inflammatory actions.

1. Introduction

Asthma has consistently been reported as a major cause of chronic cough [1]. The development of noninvasive assessment of airway inflammation led to the identification of a condition that manifests chronic cough in individuals without the abnormalities of airway function that characterize asthma, but with sputum eosinophilia. This condition was named nonasthmatic eosinophilic bronchitis (NAEB) [2]. The reason for the absence of airway hyperresponsiveness in the NAEB remains unclear. Inflammation of the airways, with recruitment and activation of T lymphocytes, eosinophils, and mast cells and release of inflammatory mediators, plays an important role in the pathophysiology of asthma and NAEB. Among lipid mediators, PGE_2 is a mediator thought to have an important role.

This paper shall summarize our current knowledge of the role of PGE_2 in lung and in respiratory disease such as asthma and nonasthmatic eosinophilic bronchitis.

2. PGE_2 Biosynthesis

Several studies suggest that prostaglandins may play an important role in orchestrating interactions between different cells in several inflammatory diseases such as asthma and rheumatoid arthritis.

Although the term prostaglandin was coined in the 1930s by Von Euler [3], and Bergstrom and Samuelsson defined the structure of two first prostaglandins in 1960 [4], the full structure of prostaglandins was not identified until 1965 by Orloff. Prostaglandins are arachidonic acid (AA) metabolites which result from enzymes with cyclooxygenase (COX) activity [5]. These metabolites are small lipidic molecules implicated in the regulation of many different processes in the organism. Their production begins with the liberation of AA from membrane phospholipids by phospholipase A2 in response to inflammatory stimuli [6]. AA is then transformed into prostaglandin H2 (PGH_2) by COXs, which is the first step in eicosanoid biosynthesis. PGH_2 is an unstable molecule that is transformed into several biologically active prostaglandins through specific enzymes with different tissue and cellular expression pattern [7].

Two isoforms of COX have been identified, and they are classified as COX-1 and COX-2. The main differences between them are their expression regulation and tissue distribution. In terms of expression, COX-1 is constitutively

expressed in cells in which prostaglandins exert physiological functions, while COX-2 expression is enhanced after inflammatory stimuli [8], such as LPS, several proinflammatory cytokines (tumor necrosis factor-alpha, interleukin-1-beta), growth factors, or tumoral promoter as PMA [4, 9]. Both isoforms catalyze similar reactions although they are codified by different genes [10]. COX-1 is associated with immediate biosynthesis of prostaglandins (several minutes after stimulation) which develop homeostatic functions; COX-2 is linked to delayed biosynthesis of prostaglandins (several hours after stimulus) which exert pathological effects. Other difference is the cellular localization; thus, COX-1 is expressed in endoplasmic reticulo, whereas COX-2 is situated in perinuclear membrane [7, 11].

PGE_2 is the most abundantly produced prostanoid in the body and has been shown to play an important role in regulating inflammatory processes. PGE_2 production is largely dependent upon three enzymatic reactions: generation of arachidonic acid from membrane glycerophospholipids via phospholipase A2, conversion of AA to an unstable intermediate prostanoid (PGH_2) by COXs, and metabolism of prostaglandin H_2 to prostaglandin E_2 via prostaglandin E synthase [12].

There are three enzymes that catalyze PGE_2 generation starting from PGH_2, namely, membrane-bound PGES (mPGES)-1 [13], mPGES-2 [14], and cytosolic PGE (cPGES) [15] which constitute two biosynthetic pathways to PGE_2 secretion; on one hand, COX-1/cPGES, the pathway implicated in homeostasis and does not play an important role in PGE_2 production [16], and on the other, COX-2/mPGES-1, the pathway associated with delayed response, inflammation, and pathology [17], and that is essential for basal production in some organs as brain, spleen, and stomach (at least in mice) [18].

Major cellular sources of PGE_2 include epithelial cells, endothelial cells, airway smooth muscle, and monocytes/macrophages [19].

3. PGE_2 Receptors

PGE_2 exerts its effects by acting on a group of rhodopsin-type G-protein-coupled membrane receptors (GPCRs) termed E-prostanoid (EP) receptors. There are four GPCR subtypes: EP1, EP2, EP3, and EP4 [20, 21]. Expression regulation of the various subtypes of EP receptor by several agents, such as inflammatory stimuli, or even PGE_2 itself, enables PGE_2 to affect tissues in a very specific and diverse manner [22, 23]. These subtypes of EP receptor differ in the intracellular signaling [24]. The expression or a combination of receptors, each which may be differentially expressed in a number of tissues (i.e., airway smooth muscle, neurons, or immune effector cells), results in a specific physiological response. These receptors could be classified according to their intracellular signaling and second messenger. EP1 receptor activation leads to an increase in intracellular calcium, usually coupled to Gq protein, and its stimulation is linked to phospholipase C [25]. EP2 and EP4 receptors couple to Gs, and activation of these receptors results in stimulation of adenylyl cyclase and increases intracellular cAMP [26]. The major signaling pathway described for the EP3 receptor is mediated by Gαi and leads to a reduction in intracellular cAMP levels. However, several EP3 receptor isoforms generated by alternative splicing from the single EP3 receptor gene have been identified, and the intracellular signal may differ [27]. Some of these isoforms of the EP3 receptor coupled to multiple G proteins produced either inhibition of adenylate cyclase and calcium mobilization or stimulation of adenylate cyclase activity [26]. Thus, accumulation of cAMP promoted by EP2 and EP4 receptors is associated with the inhibition of effector cell functions; however, EP1 and some isoforms of the EP3 receptor that increase intracellular calcium could be linked to promotion of cellular activation [20].

In pathological conditions, the role of these receptors is determined by pattern expression, ligand affinity, and their differential coupling to transduction signaling pathways in which the cellular context of the receptor is very relevant.

4. PGE_2 Effects on Respiratory System

PGE_2 is almost ubiquitous in humans and evokes potent diverse actions. It regulates several functions in the major human systems, including the gastrointestinal, reproductive, neuroendocrine, and immune systems [28].

It is in the area of inflammation that the actions of PGE_2 are most diverse because of the many specialized cell types as well as the complex and sometimes seemingly opposing actions that make that PGE_2 one of the most heterogeneous eicosanoids. This complexity reflects differences between endogenous formation and action *versus* pharmacological and exogenous addition of PGE_2 *in vivo* and *in vitro* [29].

PGE_2 acts like a pleiotropic prostaglandin with stimulating or inhibiting properties and could be classified as a regulatory element of immunity [30]. An example of this stimulating/inhibiting polarity is the opposite actions that PGE_2 exerts in the cardiovascular and immune system, where PGE_2 is able to enhance the inflammation caused by leukotrienes as well as inhibit the release of mediators and regulate monocyte macrophages and dendritic cells [31].

Although PGE_2 acts in multiple systems, we will focus our attention on the role that this prostaglandin plays in the inflammatory process linked to respiratory system.

PGE_2 is the major metabolite in the lower respiratory tract. In this system, epithelium and airway smooth muscle are the principal source of PGE_2 [32], though fibroblast, alveolar macrophages, and pulmonary endothelial cells also produce it [33, 34]. PGE_2 is important because of the multiplicity of its effects on immune response in respiratory diseases.

PGE_2 is commonly presumed to be a proinflammatory mediator and has been implicated in several inflammatory disease conditions, including rheumatoid arthritis [35]; however, PGE_2 has protective effects in different organs, and respiratory system appears to be one of them in that PGE_2 has beneficial effects [26, 36–38].

During the 1970s, PGE_2 was shown to protect against bronchoconstriction produced by ultrasonically nebulized distilled water [39] and exercise-induced asthma [40]. In the early 1990s, Pavord et al. [41] showed that inhaled

PGE_2 protected against bronchial hyperreactivity to sodium metabisulphite in which bronchoconstriction is thought to be neurally mediated. In this study, furosemide protected against bronchoconstrictor challenges in asthma, and this effect may be mediated through PGE_2. Multiple subsequent studies have observed the bronchodilator effect of PGE_2 in normal subjects [42] and patients with asthma and chronic bronchitis [43], showing that PGE_2 attenuates bronchoconstriction, possibly inhibiting the release of the bronchoconstrictor mediators which are responsible for exercise bronchoconstriction. This prostanoid inhibits early and late allergen-induced bronchoconstriction, increasing the relaxation of airway smooth muscle and inhibiting the release of mast-cell mediators and the recruitment of inflammatory cells [34]. Moreover, PGE_2 also decreases or inhibits the accompanying bronchial hyperresponsiveness to methacholine [36].

All these positive effects of PGE_2 are mainly mediated through EP2 and EP4 receptors [44]. PGE_2 can mediate bronchodilation via the EP2 receptor [45] and also anti-inflammatory effects via the EP2 and/or the EP4 receptor [46]. Also, EP2 plays an important role in aspirin-intolerant asthma because a reduction in released PGE_2 and lower expression of its EP2 receptor provoked an increase in inflammatory process in the airways of these patients [47]. However, PGE_2 also induces irritation of the upper airway, resulting in a reflex cough and enhancing the response to capsaicin. The coughing induced by this prostanoid is caused mainly, if not solely, by activation of the EP3 receptor, and in bronchoconstrictor effects are implicated by both EP1 and EP3 receptors [48].

5. PGE_2 and Inflammatory Cells

Immune cells produce a variety of prostaglandins that have both proinflammatory and anti-inflammatory effects [49]. Eosinophils are one of predominant inflammatory cells in the lungs of asthmatic patients, and changes in the number and degree of lung eosinophils probably influence disease severity. Peacock and colleagues demonstrated that PGE_2 suppresses eosinophil apoptosis, and this is likely mediated by interaction with the EP2 receptor subtype in eosinophils [50], by contrast, misoprostol (a PGE_2 analogue) inhibits eosinophil survival *in vitro* [51]. Nevertheless, in recent years, all studies have shown a negative regulator role of PGE_2 on eosinophils. This prostanoid directly controls eosinophils' migration on the cellular levels and is highly potent and efficacious. This effect is brought about mainly by EP2 receptor involving PI3K- and PKC-dependent pathways [52]. Furthermore, PGE_2 not only attenuates eosinophil trafficking but also abolishes the production of reactive oxygen species, Ca^{2+} responses, and upregulation of adhesion molecules through the EP4 receptor [53]. EP4 does not seem to depend on activation of the adenylyl cyclase/PKA pathway. Its stimulation causes phosphorylation of extracellular signal-regulated kinases (ERKs) through a PI3K-dependent mechanism [53]. So, an alternative EP2/EP4 signaling pathway in which both PI3K and PKC activation are implicated has been postulated.

PGE_2 also acts on T cells and alveolar macrophages. It is able to decrease proliferation of lymphocytes, subsequently decreasing the production of Th2 cytokines [54]. In another study, PGE_2 produced an increase of IL-10 expression, an important regulatory cytokine [55]. In contrast, PGE_2 positively controls the regulatory T cells (Treg) which are pivotal in suppressing immune responses and maintaining tolerance. PGE_2 upregulates FOXP3 mRNA and protein expression and enhances FOXP3 promoter activity [56]. PGE_2 also enhances Ig class switch to IgE in B cells [57].

PGE_2 is a route through which airway epithelial cells (AECs) modulate specific cellular subtypes such as dendritic cells. Along these lines, Schmidt and colleagues [58] demonstrated that epithelial cells, through the constitutive secretion of PGE_2, drive DCs to adopt an anti-inflammatory phenotype in an EP4 receptor-dependent manner coupled to cAMP production. As a result, PGE_2-EP4 receptor signaling generates DCs with reduced proinflammatory properties (decreased production of TNF-α and enhancing IL-10 secretion), thereby limiting DC activation [58].

Also, in alveolar macrophages, it produces an increase of IL-10 production and a decrease of TNF-α levels generated by alveolar macrophages [59, 60].

6. PGE_2 and Lung Structure

As mentioned above, the principal sources of PGE_2 in airways are the epithelial, endothelial, fibroblast, and smooth muscle cells. The epithelium is the first barrier to protect against injury. Its cells create an anti-inflammatory microenvironment that modulates the phenotype of local antigen-presenting cells (APCs), regulating the activation of local professional immune cells. A chronic state of inflammation and wound healing exists in the lung as a part of asthma pathology. Moreover, fibroblasts actively migrate and proliferate, synthesize and secrete extracellular matrix components, and differentiate into myofibroblasts. In this process, PGE_2 exerts significant negative regulatory functions by inhibiting fibroblast migration and chemotaxis with a dominant role of EP2 receptors in the cAMP-dependent inhibitory effects [61, 62], thereby decreasing fibroblast proliferation through Epac-1 and subsequent Rap1 activation (cAMP effectors) in a manner which is independent of ERK1/2 participation [63]; furthermore, PGE_2 inhibits collagen expression by a PKA-mediated process which is linked to EP2 and EP4 receptors [63]. Impaired *ex vivo* COX-2 function and PGE_2 synthesis that characterize fibroblast during the evolution of airway fibrosis may likewise promote fibrogenesis [64]. However, this inhibition of fibrosis may also occur through an inhibition of fibroblast PAR1 expression, by PAR2-mediated generation of PGE_2, one of the protease-activated receptors coupled to G protein that possesses a unique mechanism of activation and induces COX activation in a variety of cell types [65]. PAR-1 signalling is a well-known profibrotic pathway [66], so inhibition of PAR1 expression diminished pulmonary fibrosis.

PGE_2 has complex effects on airway tone, and the existence of several E-prostanoid receptors, each one with different signalling characteristics, has provided a possible

explanation for the seemingly contradictory actions of this lipid mediator. Potent relaxant effects on airway smooth muscle have been observed; however, human studies with aerosolized PGE_2 have demonstrated inconsistent effects on airway tone, with most asthmatics showing a bronchodilator response but some developing profound bronchoconstriction requiring beta agonist rescue [36, 41, 43, 48].

7. Asthma and Nonasthmatic Eosinophilic Bronchitis

Asthma is a complex chronic disease of the airways that has been estimated to affect over 300 million people worldwide, and the burden is likely to rise in the coming decades [64, 67].

The inflammatory process in asthma is characterized by inflammatory cells, mainly eosinophils, mast cells, basophils, macrophages, and Th2 cells. These cells are involved in the development of another hallmark of asthma as airway hyperresponsiveness (AHR), reversible airway obstruction, cough, mucus secretion, and structural changes by releasing inflammatory mediators such as cytokines, chemokines, growth factors, and chemical and lipid mediators [68, 69]. The complex pathogenesis of asthma is contributed to by various cellular responses, based on the dysregulated interaction between innate and adaptative immune systems.

Chronic cough is a common reason for referral to a specialist, and asthma has consistently been reported among the most common causes of chronic cough, accounting for about 25% of such cases in adult nonsmokers [1, 70, 71]. The development of sputum induction has provided a safe noninvasive method of assessing airway inflammation**.** One of the most interesting early observations made using this method was the identification of a group of patients with sputum eosinophilia identical to that seen asthma, but with normal airway function. The physiological features of this condition were different from those of asthma, and Gibson and colleagues suggested that the new disease should be known as eosinophilic bronchitis [2]. The cough in patients with nonasthmatic eosinophilic bronchitis (NAEB) responds well to inhaled corticosteroids, though the same is not the case for cough in patients without sputum eosinophilia. NAEB is responsible for about 10% of cases of isolated chronic cough referred for specialist investigation [72].

The etiology of asthma and NAEB is usually unknown, although both can be associated with exposure to an occupational sensitizer or to common inhaled allergens [73–75]; thus, the triggers that cause eosinophilic bronchitis without asthma are similar to the triggers of eosinophilic bronchitis with asthma.

In patients with eosinophilic bronchitis, there is a clear dissociation between sputum eosinophilia and airway hyperresponsiveness. The pathophysiology of eosinophilic bronchitis and the reason for the absence of airway hyperresponsiveness in this disease remains unclear. One possible explanation is that the eosinophilic airway inflammation is less active than in asthma, with less release of effector mediators. Several studies using different techniques to assess airway inflammation have shown that the inflammatory component is very similar in patients with asthma and NAEB [76]. Indeed, asthma and eosinophilic bronchitis share many immunopathologic features including increased number of eosinophils and mast cells in the superficial airway. In addition to airway eosinophilia, both conditions are associated with reticular basement membrane thickening and similar number of subepithelial T lymphocytes, mast cells, and macrophages [76]. Eosinophilic bronchitis is a disease characterized by increased expression of Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) [77, 78]. These data point to a dissociation between T-cell activation and the abnormalities in airway physiology that characterize asthma [79]. Such results suggest that Th2-mediated cytokines are closely linked to eosinophilic inflammation, though they are not necessarily associated with the physiologic hallmarks of the asthmatic phenotype.

One aspect of the inflammatory response that might be particularly important is the localization of mast cells in the airway wall, since they are present within the airway smooth muscle in asthma but not in NAEB [80]. Moreover, in subjects with eosinophilic bronchitis, CXCL8 and CXCL10 concentrations were elevated in airway secretions. These chemokines may play a key role in mast cell recruitment to the superficial airway in this condition [81]. Siddiqui and colleagues have demonstrated that mast cells are microlocalized within the airway smooth muscle bundle in asthma, which is associated with airway hyperresponsiveness [82]. Mast cells produce a variety of mediators that may interact with bronchial smooth muscle and subsequently become hyperresponsive to constrictive stimuli and proliferation [83].

8. PGE_2 in Asthma and NAEB

We recently found that induced sputum prostaglandin E2 (PGE_2) concentrations are strikingly increased in subjects with NAEB as compared with asthmatic and healthy subjects [78]. This study illustrates that, like asthma, there is active airway inflammation in eosinophilic bronchitis with release of different inflammatory mediators. These data suggest that the differences in airway function observed in subjects with NAEB and asthma may be due to differences in PGE_2 production.

Brightling et al. [84] also found numerically higher levels of PGE_2 in sputum from patients with eosinophilic bronchitis, though the differences were not statistically significant as compared with asthma patients. Recently, elevated sputum PGE_2 concentrations have been found in all patients with chronic cough [73], although in this study cough variant asthma and eosinophilic bronchitis patients were included in the same group.

The differences in sputum PGE_2 concentration between asthmatic and eosinophilic bronchitis patients may be the result of the implication of a different degradation kinetic or activity of enzymes in the synthesis and/or degradation of products of arachidonic acid metabolism. It has been recently shown that Th2 cytokines have specific effects on PGE synthase 1 and 15-PGDH enzymes in airway human epithelial cells decreasing PGE_2 [85].

We postulate that PGE_2 elevation in patients with eosinophilic bronchitis may have protective effects against the development of bronchial hyperresponsiveness. The fact that PGE_2 and its analogue have a number of bronchoprotective and anti-inflammatory effects *in vitro* [37] or after inhalation [36], as well as on allergen-induced airway responses and airway inflammation in atopic asthma [38, 86], would support such a protective role. An imbalance in the ratio of bronchoconstrictor (LTC_4) and bronchoprotective (PGE_2) lipid mediators may have a role in the pathogenesis of eosinophilic bronchitis.

PGE_2 can perform contrasting activities, which can thus lead to bronchodilation and act as anti-inflammatory or proinflammatory mediator substances in the lung [26]. Recently, several studies showed that the PGE_2 protective actions were mediated in large part by the EP_2 receptors [87, 88]. This protective action of PGE_2 might not only result in a direct effect on airway smooth-muscle relaxation, but also in the inhibition of many inflammatory processes [89]. These data may explain why patients with eosinophilia have normal tests of variable airway obstruction and airway responsiveness and experience chronic cough due to high PGE_2 levels [90].

The pathogenesis of eosinophilic bronchitis might be the opposite of that observed in aspirin-induced asthma. There is increasing evidence that, by inhibiting cyclooxygenase-1, the protective effect exerted by endogenous prostaglandin E_2 on leukotriene generation by mast cells, eosinophils, and macrophages in the airways is removed in aspirin-induced asthma. In these patients with aspirin-induced bronchoconstriction, a low production of PGE_2 has been observed, seemingly due to deficient COX-2 regulation and increased expression of leukotriene-C4 synthase [91].

9. Role of PGE_2 in NAEB

Muscle hypertrophy and hyperplasia are characteristics of asthmatic airways, and this increased layer of bronchial smooth muscle contributes to AHR in asthmatic patients [92, 93]. We hypothesized that high PGE_2 levels in the lower airways of NAEB patients constitute the essential regulatory mediator causing inhibition of bronchial smooth muscle cell proliferation (BSMC) and subsequent hyperresponsiveness [94]. Thus, we demonstrated that when BSMCs are cultured with induced sputum supernatant from NAEB patients, a strong inhibition of muscle cell proliferation takes place [94]. This inhibition was not due to the apoptotic effect of sputum supernatants or to differences in cytokine levels found in sputum. Formal proof of this finding was the addition of EP2 and EP4 antagonists to the culture recovery of the proliferation of smooth muscle cells. The lesser inhibition obtained from sputum of asthmatic patients may be explained by the absence or defectiveness of PGE_2 production, as well as by differential EP2 and EP4 receptor expression from different pathologies. Similarly, Lundequist and colleagues recently described a role for PGE_2 in protecting the pulmonary vasculature from remodeling dependent on more than one EP receptor [95].

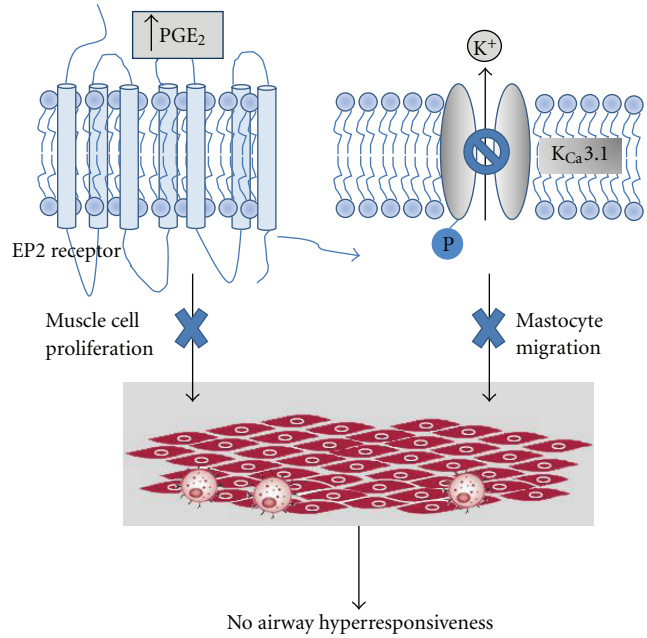

Figure 1: Hypothetical mechanisms through which PGE_2 reduces the AHR and in NAEB. (a) The PGE_2 decreases the smooth muscle proliferation producing a reduction of muscular hyperplasia, via EP2 and EP4 receptors; (b) The PGE_2 closes the K_{Ca} 3.1 channel, preventing the migration of mastocytes by means of EP2. Both mechanisms will decrease or inhibit airway hyperresponsiveness, a relevant hallmark of asthma.

An alternative explanation of the differences in airway function between asthma and NAEB is the different localization of mast cells within the airway wall. Siddiqui and colleagues have demonstrated that mast cells are microlocalized within the airway smooth muscle bundle in asthma, and this is associated with AHR [82]. Mast cells produce a variety of mediators that may interact with BSM and subsequently become hyperresponsive to constrictive stimuli and proliferation [83]. We hypothesize that both mechanisms may act synergistically (Figure 1). Recently, Duffy and colleagues reported that the engagement of the EP2 receptor closes the K^+ channel K_{Ca} 3.1 in human lung mast cells and attenuates their migration [96]; thus, PGE_2 present in sputum supernatant from NAEB patients could close the K_{Ca} 3.1 channel and inhibit mast cell migration to the airway wall and subsequently bring about microlocalization within BSMC. Furthermore, in the inhibition of BSM proliferation produced by PGE_2, the K^+ channel K_{Ca} 3.1 may be implicated since activated K^+ channels regulate human airway smooth muscle proliferation [97].

Airway smooth muscle cells are the major effector cells regulating bronchomotor tone in response to several mediators [98]. Some authors have reported that increased vascularity, reticular basement membrane thickening, and increased airway smooth muscle mass are features of both diseases [99, 100]. However, the same authors have recently reported that patients with asthma had airway wall thickening, as opposed to subjects with NAEB, who

maintained airway patency without wall thickening [101]. In addition, AHR and altered airway geometry were found to be correlated in asthma patients. Maintained proximal airway patency in NAEB compared to the subjects with asthma may protect against the development of AHR. In line with this, Park et al. have reported that proximal airway wall thickening is not a feature of NAEB [102].

In conclusion, PGE_2 present in induced sputum supernatant from NAEB patients decreases BSMC proliferation, probably due to simultaneous stimulation of EP2 and EP4 receptors with inhibitory activity. This protective effect of PGE_2 may not only be the result of a direct action exerted on airway smooth-muscle proliferation but also may be attributable to the other anti-inflammatory actions. Thus, PGE_2 agonist receptors may become a novel therapeutic approach for inflammatory respiratory diseases.

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Clinical Study

Chemokine and Free Fatty Acid Levels in Insulin-Resistant State of Successful Pregnancy: A Preliminary Observation

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Increased insulin resistance and inflammatory action are observed in pregnancy-induced hypertension (PIH), but similar insulin resistance is observed also in successful pregnancy. To estimate insulin resistance and inflammatory activity in normal pregnancy and PIH, serum concentrations of free fatty acids (FFA; corrected with albumin to estimate unbound FFA), monocyte chemoattractant protein (MCP)-1, and high-molecular weight (HMW) adiponectin were measured in severe PIH patients with a BMI less than 25 kg/m^2 and were measured 3 times during the course of pregnancy in women with normal pregnancies. FFA/albumin, MCP-1, and HMW adiponectin concentrations were significantly higher in PIH patients than in women with normal pregnancies. The 3 measurements of FFA/albumin showed a significant increase through the course of uncomplicated pregnancies. In contrast, MCP-1 and HMW adiponectin were significantly decreased during the course of pregnancy. These results suggest that the reduced MCP-1 concentration in normal pregnancy may be a pathway to inhibit the induction of pathological features from physiological insulin resistance and homeostatic inflammation.

1. Introduction

Pregnancy-induced hypertension (PIH), a leading complication in pregnancy that affects the mother and fetus, is becoming more frequent mainly because of increasing maternal age [1]. Maternal obesity, a basal condition that increases the risk of PIH 3-fold [2], has also increased over the decades [3]. On the other hand, increased insulin resistance is observed in PIH as well as in successful pregnancies [4–6]. In the course of a normal pregnancy, insulin resistance is correlated with increased maternal adipose tissue deposition [5] and supports placental formation and fetal growth.

Although the placenta is a large producer of cytokines during pregnancy [7], adipose tissue is regarded as the main organ producing insulin resistance and related cytokines [5, 6, 8, 9]. Recently, free fatty acids (FFA; also known as nonesterified fatty acids (NEFA)) were shown to be mediators of immune and inflammatory actions in adipose tissue [6, 8–13]. Although increased circulating FFA have been observed in gestational diabetes mellitus, preterm delivery, or other adverse maternal outcomes in pregnant subjects [6, 9, 14, 15], increased circulating FFA have also been described in normal pregnancy [16]. Different pathways which do not induce systemic inflammation observed in PIH [4] in insulin resistance remain unclear.

In this study, we measured peripheral monocyte chemoattractant protein-1 (MCP-1), a proinflammatory chemokine that induces monocyte action leading to cell adhesion and endothelial dysfunction, FFA, and high-molecular weight (HMW) adiponectin, a major adipocytokine that reflects insulin sensitivity, in PIH patients and made repeated measurements of these molecules in women with normal pregnancies throughout the course of pregnancy. We hypothesized that an alteration of the chemokines in the inflammatory pathway protects women with normal pregnancies, but not PIH patients, from cardiovascular disorders in a state of physiological insulin resistance.

2. Materials and Methods

2.1. Subjects. This study was reviewed and approved by the Institutional Review Board of Nara Medical University, and

informed consent was obtained from each subject. For the preliminary study, we recruited 17 nonpregnant women with body mass index (BMI) under 25 kg/m^2, 25 normal pregnant women at 28 weeks or later of gestation, and 7 severe PIH patients. The pregnant women had BMIs under 25 kg/m^2 prior to pregnancy. All women were East Asian, and none were taking any medications or showed evidence of any metabolic diseases or complications other than PIH. Severe PIH was defined as the new onset of 2 consecutive measurements of diastolic blood pressure ≥110 mmHg and systolic blood pressure ≥160 mmHg diagnosed after 20 weeks of gestation. After the preliminary study, we recruited 36 normal pregnant women for sample correction by taking measurements 3 times throughout the course of pregnancy (1st screening = around 12 weeks of gestation; 2nd = 28 weeks; and 3rd = 36 weeks) for a longitudinal study and paired analysis. All subjects had serum samples available for analysis and did not have gestational diabetes mellitus, thyroid malfunction, or other complications except hypertension. Proteinuria was not considered within the criteria of this study.

All venous blood samples were obtained after an overnight fast at routine medical examination. Serum was separated immediately and stored at −80°C for 3 years for the longest and 6 month for the shortest storage.

2.2. Enzyme Immunoassays. Serum FFA (mainly palmitic acid) were measured in duplicate with a commercially available kit (BioVision Research Products, Mountain View, CA). The lower limit of detection was 2 μM. To estimate alterations in unbound FFA, the data were corrected with serum albumin concentrations (FFA (μM)/albumin (g/dL)) by using the BCG albumin assay kit (BioChain, Hayward, CA). The lower limit of detection of albumin was 0.01 g/dL. Serum HMW adiponectin level and its ratio to total adiponectin were measured on the same 96-well plate in duplicate using a commercially available protease-pretreated ELISA kit (Sekisui Medical, Co., Ltd., Japan). The lower limit of detection was 0.075 ng/mL. The intraassay coefficient of variation (CV) was within ±20%, while the inter-assay CV was not more than 15%. Serum leptin and MCP-1 concentrations were measured in duplicate with commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN). The lower limit of detection was less than 7.8 pg/mL for leptin and less than 5.0 pg/mL for MCP-1. For leptin, the intraassay CV was 3.3% at a concentration of 64.5 pg/mL, 3.0% at 146 pg/mL, and 3.2% at 621 pg/mL, while the inter-assay CV was 5.4% at 65.7 pg/mL, 4.2% at 146 pg/mL, and 3.5% at 581 pg/mL. For MCP-1, the intraassay CV was 7.8% at a concentration of 76.7 pg/mL, 4.7% at 364 pg/mL, and 4.9% at 1121 pg/mL, while the inter-assay CV was 6.7% at 74.2 pg/mL, 5.8% at 352 pg/mL, and 4.6% at 1076 pg/mL. In the longitudinal study, measurement of FFA, albumin, MCP-1, and HMW adiponectin were performed using the techniques described above.

2.3. Statistical Analysis for Human Serum Measurement. In the preliminary study, we compared normal pregnant women with nonpregnant women and compared PIH patients with normal pregnant women at 28 weeks or later of gestation. Statistical analysis was performed using the Mann-Whitney U-test (SPSS 15.0J; SPSS Japan Inc., Japan). In the longitudinal study, results in respective patients were analyzed in pairs using repeated measures of ANOVA with the post hoc test (Bonferroni correction; SPSS 15.0J). Statistical significance was set at $P < 0.05$. All values are expressed as the mean ±SEM.

3. Results

3.1. Preliminary Study: FFA and Other Adipocyte-Derived Inflammatory Factors in PIH. In the preliminary study, we compared normal pregnant women at 28 weeks or later of gestation with nonpregnant subjects and compared PIH patients with normal pregnant women at 28 weeks or later of gestation. Subject characteristics are shown in Table 1. Diastolic blood pressure was significantly lower in normal pregnant subjects than in the nonpregnant subjects. Blood pressure values were significantly higher in PIH subjects than in normal pregnant women.

Serum concentrations of FFA (raw data and after correction with albumin), MCP-1, total and HMW adiponectin (raw data and ratio), and leptin are shown in Table 1. FFA concentrations were significantly higher in PIH subjects than in normal pregnant women but no significant difference was observed between normal pregnancy and nonpregnant controls. However, after the albumin correction (an estimated value reflecting unbound FFA), serum concentrations were significantly higher in normal pregnancy than in nonpregnant controls. Serum concentrations of MCP-1 were significantly lower in normal pregnant subjects than in nonpregnant controls and were significantly higher in PIH than in normal pregnant subjects. HMW adiponectin concentration and its ratio to total adiponectin were significantly lower in normal pregnant subjects than in nonpregnant subjects and were higher in PIH subjects than in normal pregnant women. Serum leptin was significantly increased only in PIH patients compared to that in normal pregnant subjects. These trends in HMW adiponectin, leptin, and MCP-1 are similar to those in our former report that included subjects with BMIs greater than 25 kg/m^2 [17]; however, the trends in total adiponectin and HMW-to-total adiponectin ratio differ from those in our previous report.

3.2. Longitudinal Study: FFA, MCP-1, and HMW Adiponectin during the Course of Normal Pregnancy. Subject characteristics are shown in Table 2. FFA concentrations were not significantly altered in normal pregnant women over the course of 3 measurements: 1st screening, 97.47 ± 13.05 μM; 2nd, 110.89 ± 12.78; 3rd, 120.85 ± 12.24. However, after the albumin correction (1st screening, 3.15 ± 0.04 g/dL; 2nd, 2.57 ± 0.03; 3rd, 2.52 ± 0.03) to estimate the alteration of unbound FFA, the value (FFA [μM]/albumin [g/dL]) was significantly increased throughout the course of pregnancy: 1st screening, 31.38±4.29; 2nd, 42.51±4.82; 3rd, 48.45±5.10; $P = 0.0048$ (Figure 1). In contrast, MCP-1 concentrations decreased significantly during the course of pregnancy: 1st screening, 154.36 ± 20.27 pg/mL; 2nd, 110.56 ± 33.44; 3rd,

Table 1: Characteristic of the subjects and serum concentrations of the molecules.

	Nonpregnant control	Normal pregnancy later than 28 weeks	Pregnancy-induced hypertension
n	17	25	7
Gestational age at sampling (weeks)		33.2 ± 0.7	32.3 ± 0.8
BMI at sampling (kg/m^2)	20.3 ± 0.4	23.9 ± 0.5*	24.5 ± 0.6
BMI before pregnancy (kg/m^2)	20.3 ± 0.4	20.5 ± 0.4	20.9 ± 0.9
Blood pressure			
Systolic	106.3 ± 1.8	107.3 ± 1.8	174.8 ± 2.0
Diastolic	73.1 ± 1.2	58.8 ± 1.6*	100.3 ± 2.6**
MAP	84.1 ± 1.1	74.9 ± 1.5*	107.3 ± 17.9**
FFA (μM)	155.62 ± 29.17	153.68 ± 16.23	236.54 ± 31.89**
Albumin (g/dL)	3.48 ± 0.07	2.62 ± 0.05*	1.92 ± 0.12**
FFA/Alb	41.62 ± 8.50	58.82 ± 5.94*	124.94 ± 15.96**
MCP-1 (pg/mL)	219.50 ± 12.17	131.95 ± 8.63*	195.41 ± 27.22**
Adiponectin			
Total (μg/mL)	8.85 ± 0.62	5.33 ± 0.37*	6.82 ± 1.00
HMW (μg/mL)	4.45 ± 0.46	1.97 ± 0.20*	3.66 ± 0.62**
HMW/total adiponectin ratio	0.49 ± 0.03	0.36 ± 0.02*	0.53 ± 0.04**
Leptin (ng/mL)	6.91 ± 0.91	8.31 ± 1.09	35.56 ± 10.31**

BMI: body mass index; MAP: mean arterial pressure; FFA: free fatty acids; MCP: monocyte chemotactic protein; HMW: high-molecular weight.
*$P < 0.05$ versus nonpregnant control.
**$P < 0.05$ versus normal pregnancy later than 28 weeks.

Table 2: Characteristic of the subjects of longitudinal study.

n	36
Maternal age at delivery (years)	30.9 ± 0.7
Parity (times)	
0	18
1	13
2 or more	5
Average gestational age at sampling (weeks$^{+\text{days}}$)	
1st	11^{+5} (8^{+2}–14^{+4})
2nd	28^{+3} (27^{+2}–29^{+5})
3rd	36^{+1} (35^{+0}–37^{+2})
BMI before pregnancy (kg/m^2)	22.1 ± 0.6
BMI on delivery (kg/m^2)	26.4 ± 0.6
Average gestational age at delivery (weeks$^{+\text{days}}$)	39^{+5} (36^{+6}–41^{+3})
Infant birth weight (g)	3052.0 ± 78.7

BIM: body mass index.

108.78 ± 28.17; $P < 0.0001$ (Figure 1). HMW adiponectin was also significantly decreased during the course of pregnancy: 1st screening, 3.48 ± 0.30 μg/mL; 2nd, 2.88 ± 0.27; 3rd, 2.86 ± 0.25; $P = 0.0001$ (Figure 1).

4. Discussion

Our preliminary study showed increases of FFA (particularly bioactive unbound FFA) and MCP-1 in lean severe PIH patients. However, in normal pregnancy, FFA increased but MCP-1 significantly decreased in the serum. In longitudinal study throughout normal pregnancy, increase of FFA and the decrease of MCP-1 during the course have been clarified. A significant decrease in HMW adiponectin, which may be consistent with the physiological increase of insulin resistance in normal pregnancy, was also confirmed.

Fatty acids play pivotal roles in the development of several diseases including adult metabolic syndrome [8, 10–12] and pregnancy complications such as miscarriage [6] or preterm delivery [14], though fatty acids are also involved in the successful physiological distribution of energy in pregnancy [18]. It was recently revealed that FFA are mediators of toll-like receptor (TLR)-4 and the NF-kappaB pathway of macrophages within adipose tissue and are regarded as key molecules in systemic inflammation, which plays a role in type 2 diabetes and cardiovascular disease [10, 12, 13]. As we reviewed in this journal [19], TLRs may contribute to pregnancy pathologies. Several reports described increased FFA in preeclampsia [6, 15] but these reports did not estimate unbound FFA. In preeclamptic patients, acute inflammation is one of the major features of preeclampsia pathophysiology [2, 4, 6, 7, 15]. A difference between our study and former studies is that we chose only lean subjects, who may not show adipocyte hypertrophy. Adiponectin was increased in our PIH subjects. We recently reported that increased brain-type natriuretic peptide (BNP) correlated with increased adiponectin in PIH [17], similar to the findings in acute coronary syndrome [20] and cardiomyopathy [21]. We also showed that BNP induced

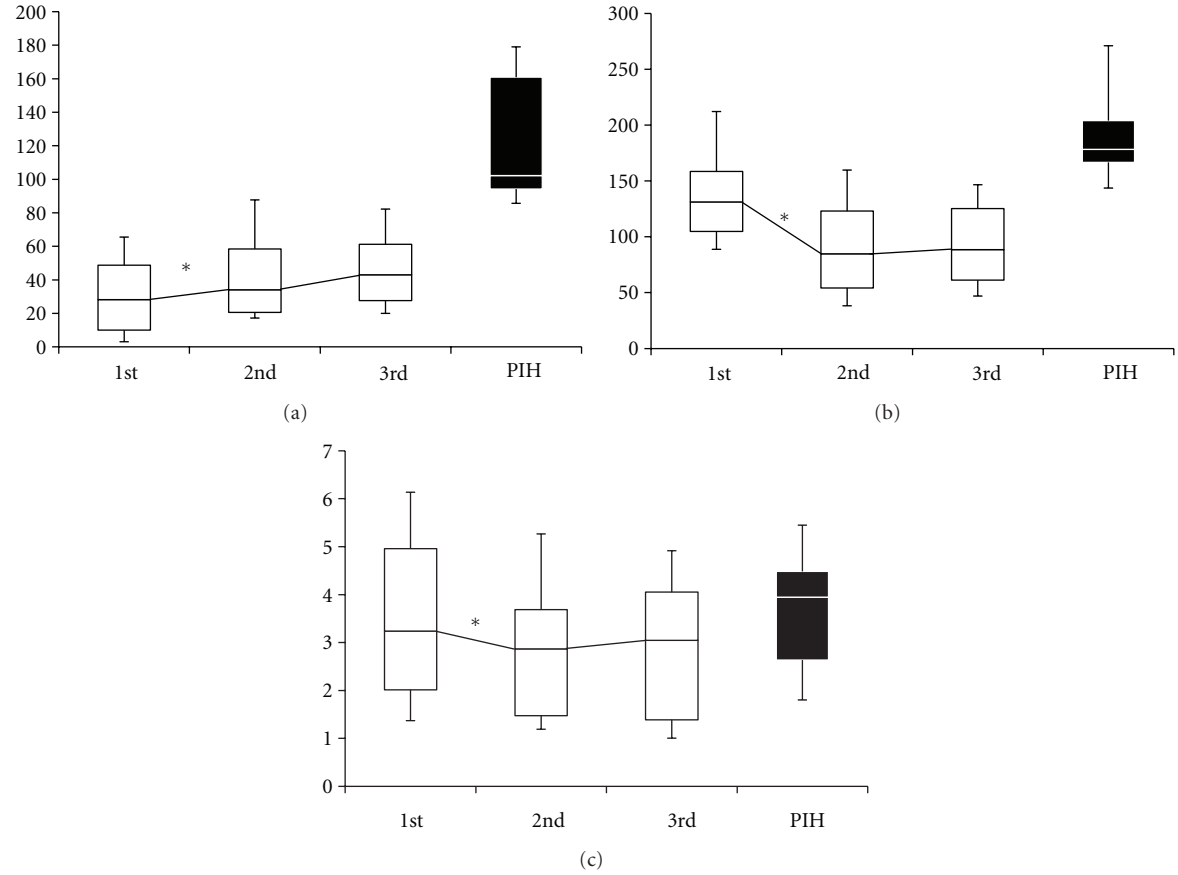

Figure 1: (a) Serum concentrations of FFA (μM)/albumin (g/dL), (b) MCP-1 (pg/mL), and (c) high-molecular weight (HMW) adiponectin (μg/mL) during normal pregnancy and PIH (for reference; see Table 1). In normal pregnancy, samplings were performed 3 times in each subject (see Table 2) and analyzed in pairs. Data are shown as the 90th, 75th, 50th, 25th, and 10th percentile of each measurement group. $^*P < 0.05$ in repeated measures of ANOVA with the post hoc test.

the release of adiponectin from cultured adipocytes *in vitro* [17]. In this respect, further research is needed to reveal the conditions of adipose tissue in PIH patients without obesity.

MCP-1 is a major chemokine and proinflammatory cytokine that activates monocyte recruitment and strongly contributes systemically to the pathology of inflammation. It is now well accepted that the insulin-resistant state induces the mitogen-activated protein kinase pathway and increases MCP-1 secretion from adipocytes [11, 12], indicating that MCP-1 potentiates the pathology of insulin resistance. Increased MCP-1 was observed in pregnant women with severe obesity [22, 23] and preeclampsia [17, 24]. Additionally, MCP-1 secretion was reported from the human early invasive trophoblast [25]. However, a peripheral decrease of MCP-1 in successful human pregnancy has only been described in a single report using multiple cytokine arrays [26], showing a similar decrease of the serum concentration of this molecule during pregnancy. This decrease may be a system adaptation in humans to avoid pathologic activation of monocytes in pregnancy-induced insulin resistance. The only evidence to support this hypothesis was reported in spontaneously hypertensive rats. MCP-1 expression was increased in the kidney in rat but expression declined significantly after the rats became pregnant, and blood pressure was also decreased [27]. This paper suggests the existence of an adaptation system during pregnancy via chemokine regulation.

The limitation of this study is that food intake was not equalized between each sampling even though all samples were taken after an overnight fast. FFA concentrations are altered for several days after different food choices, and albumin may decrease with emesis or anemia. A larger cohort study or more frequent sampling may reduce the alteration of the results after an unusual dietary event.

5. Conclusions

Although FFA were also increased during the course of normal pregnancy, which may be consistent with physiological insulin resistance, MCP-1 was decreased, which would inhibit MCP-1-mediated pathologic inflammation during the hyperlipidemic state of a successful pregnancy. FFA and MCP-1 in adipose tissue are regarded as key molecules in homeostatic inflammatory linkage. This is the first report suggesting a difference between pathological inflammation and reasonable insulin resistance in human pregnancy.

Further research in adipocytokines and adipose tissue may lead new statistics for prediction and therapy of pregnancy complications.

Abbreviations

PIH: Pregnancy-induced hypertension
FFA: Free fatty acids
NEFA: Nonesterified fatty acids
MCP-1: Monocyte chemoattractant protein-1
HMW: High molecular weight
BMI: Body mass index.

Conflict of Interests

The authors declare that they have no conflict of interests.

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