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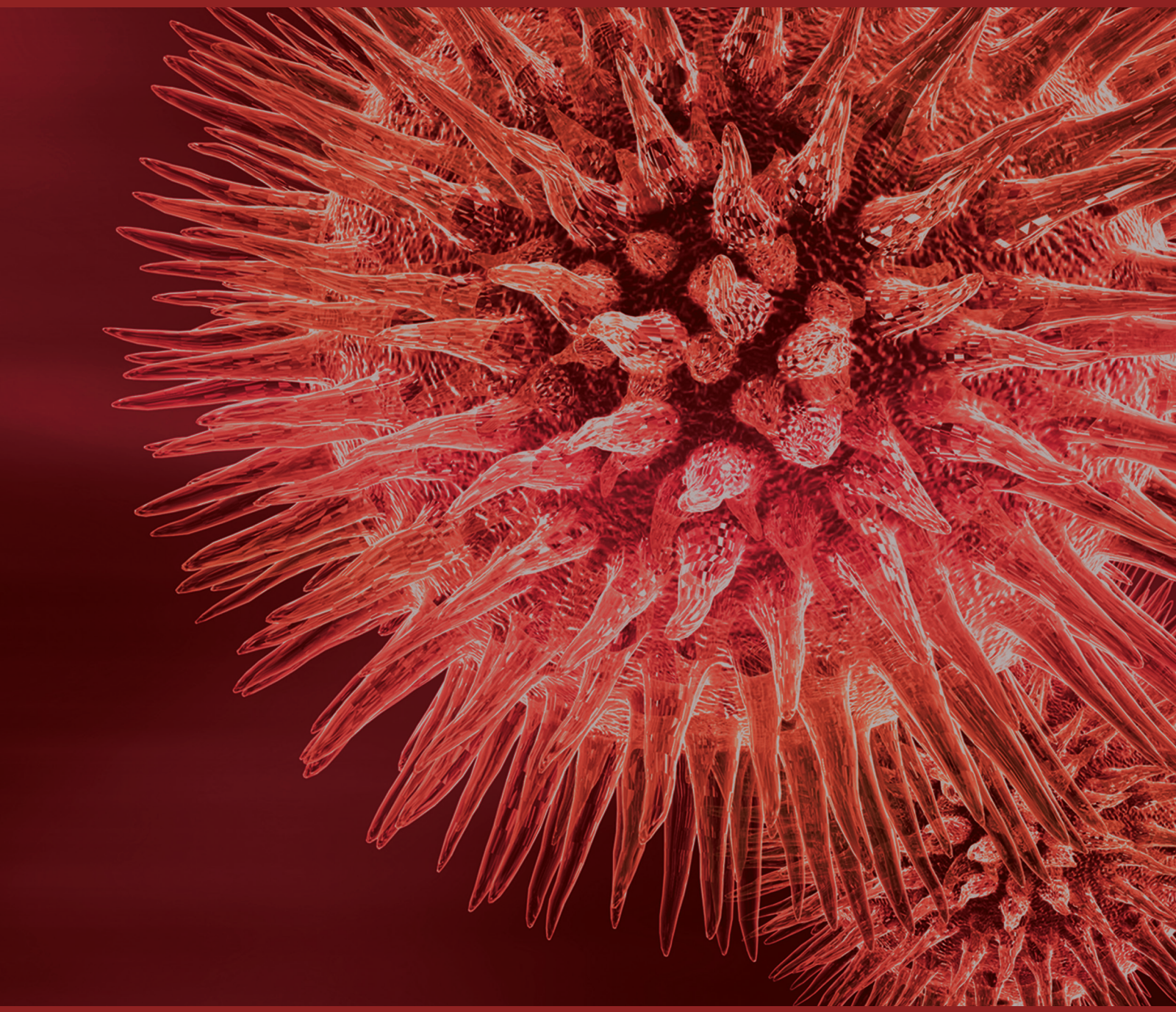
The Role of Lipids Mediators in Inflammation and Resolution

Guest Editors: Alexandre de Paula Rogerio, Carlos Artério Sorgi, Ruxana Sadikot, and Troy Carlo



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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 605959, 2 pages
http://dx.doi.org/10.1155/2015/605959

Editorial

The Role of Lipids Mediators in Inflammation and Resolution

Alexandre de Paula Rogerio,[1] Carlos Artério Sorgi,[2] Ruxana Sadikot,[3] and Troy Carlo[4]

[1]*Departamento de Clínica Médica, Laboratorio de Imunofarmacologia Experimental (LIFE), Instituto de Ciências da Saúde, Universidade Federal do Triangulo Mineiro (UFTM), Rua Vigário Carlos 162, 38025-350 Uberaba, MG, Brazil*
[2]*Faculdade de Ciencias Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, 14049-903 São Paulo, SP, Brazil*
[3]*Pulmonary, Critical Care, Sleep and Allergy Medicine, University of Florida, Gainesville, FL 32610, USA*
[4]*Pulmonary and Critical Care Medicine Division, Brigham and Women's Hospital, Boston, MA 02115, USA*

Correspondence should be addressed to Alexandre de Paula Rogerio; alexprogerio@biomedicina.uftm.edu.br

Received 8 December 2014; Accepted 8 December 2014

Acute inflammation is generally self-limited. However, if acute inflammation fails to resolve, chronic inflammation can persist. The innate and adaptive immune systems, as well as structural cells, modulate the length and intensity of inflammatory responses. Aberrant immune responses, including those induced by allergens, environmental pollutants, infectious agents, acids, and other noxious stimuli, promote excessive leukocyte recruitment and the production of proinflammatory cytokines, lipids mediators, and chemokines, which are critical to initiate and maintain the inflammatory process. Lipids mediators, derived from the omega-6 polyunsaturated fatty acids (PUFA) including leukotrienes (LTs) and prostaglandins (PGs), are potent enhancers of innate and adaptive immune activity and are implicated in numerous inflammatory disorders. Yet certain PGs, such as PGD_2 and PGE_2, demonstrate anti-inflammatory effects. Similarly, lipoxins (LXs), derived from the omega-6 PUFA arachidonic acid, not only harbor potent anti-inflammatory activity, but also promote the resolution of inflammation. Complete resolution of inflammatory responses is critical for human health. Resolution is an active process that is regulated, in part, by specialized proresolving mediators such as the omega-3 PUFA derived resolvins, maresins, and protectins, in addition to the aforementioned LXs. These biochemical mediators signal through distinct receptors to both dampen inflammation and promote resolution.

This special issue covers the most recent research elucidating the role of these lipids mediators in inflammation and the resolution of inflammation.

In recent decades, considerable progress has been made in understanding the role of lipoxin A_4 in health and disease. Two elegant reviews by Higgins et al. and Martini et al. illustrate the critical role LXA_4 plays in patients with cystic fibrosis and neurological diseases, respectively.

Cystic fibrosis, an autosomal disease, leads to, among others, devastating infection and inflammation of the airways. Patients suffering from cystic fibrosis display decreased LXA_4 production when compared to healthy individuals suggesting that this decrement contributes to continuous local inflammation. Interestingly, LXA_4 triggers responses in bronchial epithelial cells that would be beneficial to CF patients. Exposure to LXA_4 stimulates a rapid and transient intracellular Ca^{2+} increase and whole-cell Cl^- currents that restore fluid transport in cystic fibrosis, increases the airway surface liquid height via P2Y11 activation, and enhances epithelial cell migration and proliferation, activities crucial to the repair of epithelia. Thus, LXA_4 demonstrates therapeutic potential for patients with cystic fibrosis.

Neurological diseases and conditions, such as Alzheimer's, Parkinson's, traumatic brain injury, and stroke as well as conditions leading to chronic neuropathic pain, typically present marked transient or continued neuroinflammation. Interestingly, Alzheimer's patients are slow to resolve inflammation and display lower LXA_4 levels in cerebrospinal fluid and hippocampus samples compared to control subjects. Aspirin-triggered 15-epi-lipoxin A_4 promoted decreased inflammation in a murine model of Alzheimer's by reducing proinflammatory and increasing

anti-inflammatory mediators in the brain. Taken together, these demonstrate the neuroprotective properties of LXA_4.

Protozoan infections cause serious health, political, social, and economic problems. In an experimental model, Sacramento et al. demonstrated that 5-lipoxygenase knockout animals displayed increased susceptibility to infection with *Leishmania infantum* as measured by an increase in parasitic load in several organs as well as decreased neutrophil migration to the infectious foci. In addition to these effects, reductions in proinflammatory cytokines involved in T cell differentiation to Th17 axis were observed. These results demonstrated that LTs play an important role in the controlling of *L. infantum*-induced visceral leishmaniasis.

Cysteinyl leukotrienes (cysLTs), like LTs, play an important role in diseases, such as asthma. Allergic asthma is a complex inflammatory disorder characterized by airway hyperresponsiveness, eosinophilic inflammation, hypersecretion of mucus, and tissue remodeling. The asthma pathophysiology involves chemical mediators that play an important role in the establishment of inflammation. Baptista-dos-Reis et al. review the roles of cysLTs in eliciting eosinophil granule protein secretion and emphasize the importance of this finding in eosinophil immunobiology and in eosinophilic diseases.

Allergen exposure may induce changes in brainstem secondary neurons, with neural sensitization of the nucleus solitary tract, which can be considered one of the causes of the airway hyperresponsiveness, a characteristic feature of asthma. Based on these considerations, Spaziano et al. evaluated functional, morphological, and biochemical changes occurring in the nucleus solitary tract following airway sensory nerve activation in naive and ovalbumin sensitized rats.

The role of inflammation in diabetes is widely known. Tessaro et al. carefully review the roles eicosanoids play in diabetes-related nephropathy, retinopathy, and cardiovascular events.

Acknowledgment

We would like to thank the authors across the world for their valuable contributions to this special issue as well as the reviewers for their constructive comments to the manuscripts. We encourage and appreciate your further support for this annual/special issue series.

Alexandre de Paula Rogerio
Carlos Artério Sorgi
Ruxana Sadikot
Troy Carlo

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 568408, 8 pages
http://dx.doi.org/10.1155/2015/568408

Review Article

Lipid Mediators Are Critical in Resolving Inflammation: A Review of the Emerging Roles of Eicosanoids in Diabetes Mellitus

Fernando H. G. Tessaro, Thais S. Ayala, and Joilson O. Martins

Laboratory of Immunoendocrinology, Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of São Paulo, Avenida Professor Lineu Prestes 580, Bloco 17, 05508-000 São Paulo, SP, Brazil

Correspondence should be addressed to Joilson O. Martins; martinsj@usp.br

Received 13 July 2014; Revised 27 October 2014; Accepted 27 October 2014

Academic Editor: Carlos Artério Sorgi

The biosynthesis pathway of eicosanoids derived from arachidonic acid, such as prostaglandins and leukotrienes, relates to the pathophysiology of diabetes mellitus (DM). A better understanding of how lipid mediators modulate the inflammatory process may help recognize key factors underlying the progression of diabetes complications. Our review presents recent knowledge about eicosanoid synthesis and signaling in DM-related complications, and discusses eicosanoid-related target therapeutics.

1. Introduction

Eicosanoids are biologically active lipid mediators that regulate inflammation [1] and that include prostaglandins (PGs), prostacyclins, thromboxanes (TX), leukotrienes (LT), and lipoxins (LX) (Figure 1) [2–4]. They may amplify or reduce inflammation, which coordinates cytokine production, antibody formation, cell proliferation and migration, and antigen presentation [2, 5, 6]. To prevent great tissue damage, eicosanoids also control the inflammatory resolution and tissue repair process [7, 8]. Imbalances in eicosanoid synthesis have been reported to drive chronic inflammation [1, 9], which deregulates signaling pathways and/or cellular events leading to abnormal immune functions [6, 10]. In particular, circulating and local mediators, such as eicosanoids, interleukin- (IL-) 1β, tumor necrosis factor- (TNF-) α, IL-6, IL-8, macrophage migration inhibitory factor (MIF), and free radicals, create a state of low-chronic inflammation in diabetic patients [5, 10, 11]. Inflammation may lead to diabetes progression, including damage to the kidneys (diabetic nephropathy), eyes (diabetic retinopathy), nerves (diabetic neuropathy), and cardiovascular system [12] (Figure 2).

In this review, we summarize the role of eicosanoids on the pathogenesis and progression of diabetes. In addition, we review drugs used to treat diabetic complications by acting on compounds of the eicosanoid pathway and speculate on possible future targets to treat diabetes complications.

2. The Role of Eicosanoids in Diabetes

The level of inflammation severity in diabetes is associated with hemoglobin A1 levels [13]. Increased PGE_2 levels are related to dysfunction in insulin-regulated glycogen synthesis and gluconeogenesis in the liver [14, 15]. 12- as well as 15-hydroxyeicosatetraenoic acid (HETE) increases inflammatory cytokine expression, such as IL-6, TNF-α, and MCP-1, inducing chronic inflammation and the infiltration of inflammatory cells in adipose tissue [16–18]. In addition, 12-lipoxygenase (LOX) metabolites impair insulin action in adipocytes and can downregulate glucose transport, both of which may lead to insulin resistance [18, 19]. Nimesulide and metformin improved acute inflammation and impaired glucose metabolism [20], suggesting that impairing functions of prostaglandin synthesis are mediated by altered glucose levels [21].

2.1. Diabetic Nephropathy. Diabetic nephropathy is the major cause of diabetes-related death [22]. Renal disorders associated with diabetic nephropathy consist of modifications in

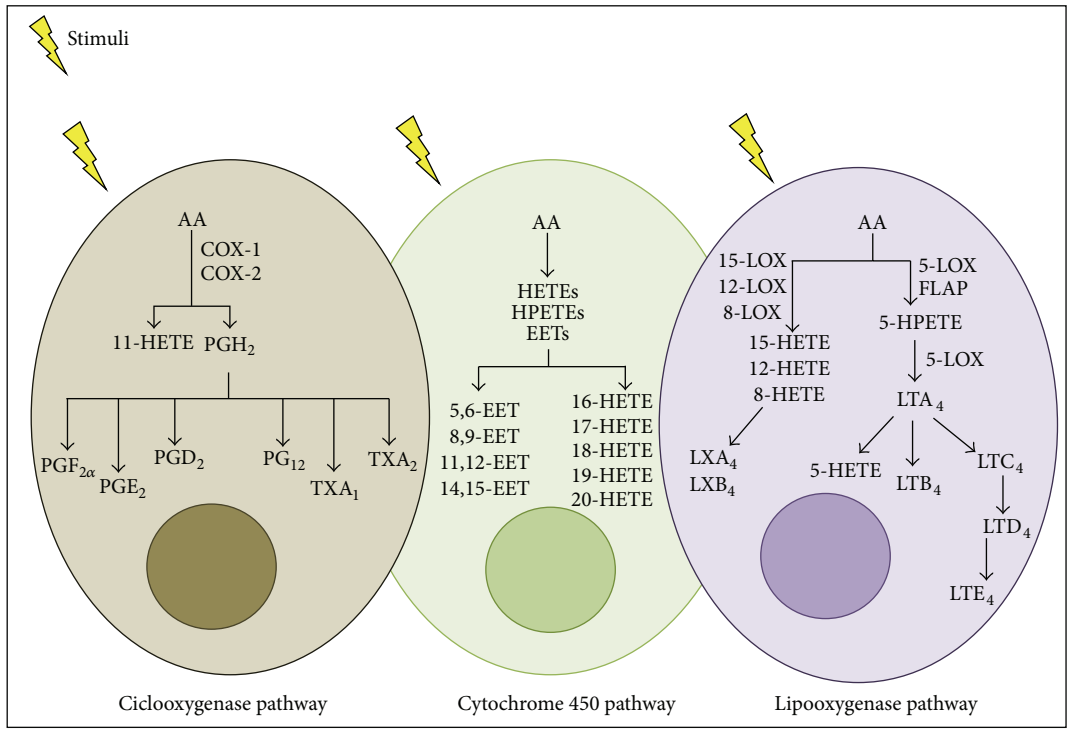

Figure 1: Eicosanoid synthesis pathways. After cell stimulation, arachidonic acid (AA) can be metabolized by three enzymes: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP 450). COX catalyzes AA in (prostaglandin) PGG_2 and PGH_2, and these are converted into PGD_2, PGE_2, $PGF_{2\alpha}$, PG_{12}, TXA_1, and TXA_2. The LOX pathway catalyzes AA into hydroxyeicosatetraenoic acids (HETEs) and diverse hydroperoxyeicosatetraenoic acids (HPETEs). This pathway involves four enzymes: 5-LOX, 8-LOX, 12-LOX, and 15-LOX. 5-LOX interacts with a 5-LOX-activating protein (FLAP), enhancing the interaction of 5-LOX to AA. LTA_4 hydrolases convert LTA_4 into LTB_4, and LTC_4 synthase can convert LTA_4 to LTC_4, whereupon it is then metabolized to LTD_4 and LTE_4. 5-LOX synthetizes LXA_4 and LXB_4 using 15-HETE. The pathway of CYP-450 leads to the conversion of HETEs, including 16-, 17-, 18-, 19-, and 20-HETE and epoxyeicosatrienoic acids (EETs): 5,6-, 8,9-, 11,12-, and 14,15-EET.

renal hemodynamics, glomerular hypertrophy, mesangial cell proliferation, matrix accumulation, and proteinuria [23]. In normal conditions, PGE_2 is the major PG in the kidneys and acts in renal physiology, glomerular filtration, and renin release [24, 25]. PGE_2 activates kidney EP receptors, such as EP1, EP2, EP3, and EP4 in the collecting duct (except for EP2 whose mRNA has been localized to the outer and inner medulla of the kidney and EP4 which can also be expressed in the glomerulus) [25, 26]. Interactions between resident renal cells and macrophages change the microenvironment to a proinflammatory state, contributing to tissue damage and scarring [27, 28]. Macrophages and T cells infiltrate the glomeruli and interstitium, contributing to chronic renal failure in diabetic patients [27, 29–31].

During inflammation, macrophages release IL-1B and TNF-α, inducing endothelial cell permeability, altering glomerular hemodynamics, and decreasing PGE_2 production by mesangial cells [32]. Normal levels of PGE_2 suppress Th1 immune responses [33] and downregulate TNF-α production and upregulate IL-10 production through EP2 and EP4 receptor signaling, ending nonspecific inflammation [33–35]. Through an IL-10-dependent mechanism, PGE_2 regulates IL-12 secretion by selectively inhibiting IL-12p70 production and stimulating IL-12p40 release [36, 37]. However, PGE_2 is reduced in diabetic nephropathy, and this plays an essential role in the evolution of diabetic renal injury, strengthening the conclusion that inflammatory mechanisms have a significant role in both diabetic nephropathy development and progression [38–40]. Knockout podocyte-specific mice are protected against diabetes-induced nephropathy and albuminuria, showing the importance of COX-2 metabolites in the establishment of diabetic nephropathy [41].

2.2. Diabetic Retinopathy. Estimates done between 2005 and 2008 suggest that 28.5% of diabetics over the age of 40 in the United States had diabetic retinopathy and vision-threatening problems [42]. Low-grade chronic inflammation has been implicated in the pathogenesis of diabetic retinopathy [43]. The retina of diabetic individuals has a particular lipid profile [44]. COX-2 increases in the retina of diabetic animals, which contributes to abnormal production of PG [45].

5-LO-derived 5-HETE is the major proinflammatory eicosanoid, being five times higher in the vitreous of diabetics versus nondiabetics patients [46]. Mice *null* for the 5-LO gene demonstrated a minor inflammatory reaction [47–49]. Mice deficient in 5-LO had significantly less degeneration of retinal capillaries induced by diabetes, less superoxide

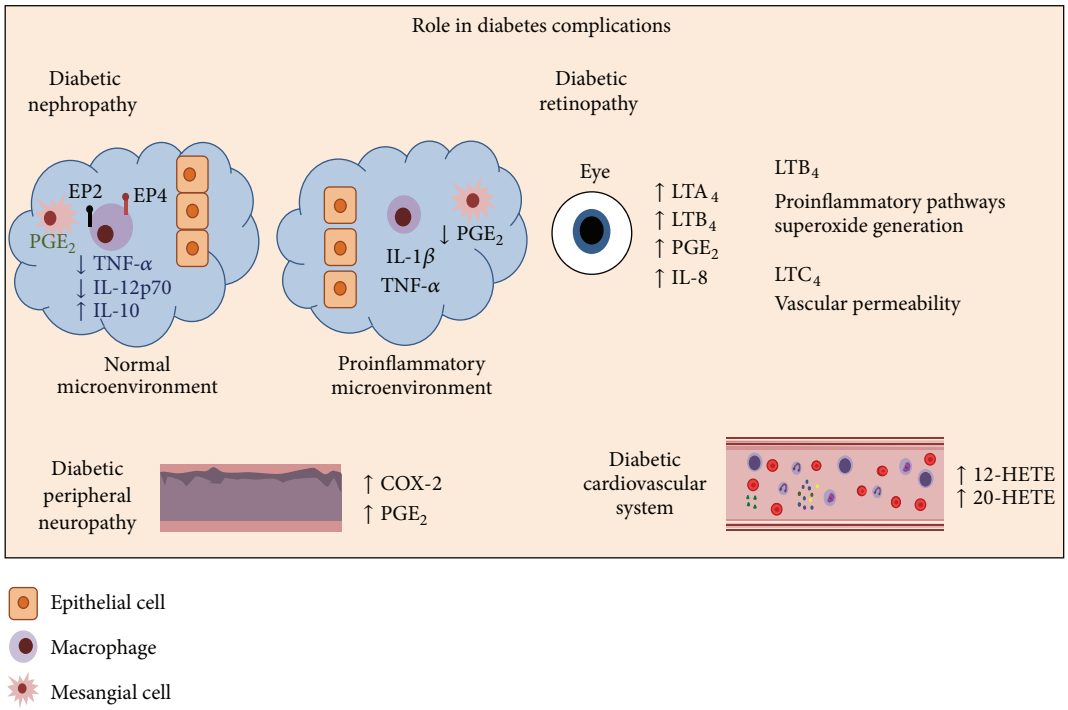

Figure 2: Eicosanoid compounds affect different organs in diabetes complications. Diabetic nephropathy, one of the most common complications in diabetes, shows low PGE_2 levels and altered glomerular hemodynamics. This dilates arteries and increases microvascular permeability. In normal conditions PGE_2 downregulates TNF-α production and upregulates IL-10 production through EP2 and EP4 receptor signaling. However, a proinflammatory environment leads to cell permeabilization, low concentrations of PGE_2, and mesangial cell proliferation. Diabetic retinopathy is another common complication in diabetes. In diabetes, the environment in the retina has a particular lipid profile, with higher COX-2 and abnormal production of PG. LTA_4 and LTB_4 are enhanced in addition to IL-8. Diabetic peripheral neuropathy is correlated with high COX-2 and PGE_2. In a diabetic's cardiovascular system, PGE_2 has an important role in microvascular permeability, and 12-HETE and 20-HETE lower the activity of endothelial progenitor cell (EPC) function.

generation, and less nuclear factor (NF)-kB expression [50]. Therefore, the generation of LTs could contribute to chronic inflammation and retinopathy in diabetes [51].

In addition, a hyperglycemic environment causes the release of 5-LO metabolites, LTA_4 and LTB_4. Retinas from both nondiabetic and diabetic mice are unable to produce LT or 5-LO mRNA. However, it was demonstrated that transcellular delivery of LTA_4, from bone marrow-derived cells to retinal cells, results in the generation of LTB_4/LTC_4 [52]. LTC_4 induces vascular permeability after binding with the retinal microvascular endothelial cells, and LTB_4 coordinates proinflammatory pathways and superoxide generation, which may contribute to endothelial cell death and capillary degeneration, in turn contributing to chronic inflammation and diabetic retinopathy development [53].

2.3. Diabetic Peripheral Neuropathy. Estimates suggest 50% of diabetic patients have diabetic peripheral neuropathy, which affects the sensorimotor and autonomic parts of the peripheral nervous system [54–56]. Few studies describe the involvement of the eicosanoid pathway in DPN. In streptozotocin-induced rats, the intrathecal administration of COX-2 inhibitors, but not of COX-1 or COX-3 inhibitors, had an antihyperalgesic effect, supporting the importance of spinal COX-2 in DPN [57]. Pain may be attributed to the action of PGE_2 on peripheral sensory neurons and on central sites within the spinal cord and the brain [58].

2.4. Diabetic Cardiovascular System. Impaired endothelial function is described in diabetes [59–61]. COX-2 expression and dilator prostaglandin synthesis increase in the coronary arterioles of diabetic patients [62]. Venous smooth muscle cells express more COX-2 and release more PGE_2 when stimulated by a mix of inflammatory cytokines [63]. PGE_2 causes pyrexia, hyperalgesia, and arterial dilation [58, 64]. PGE_2 may act as a mediator of active inflammation, promoting first local vasodilatation, then the recruitment and activation of neutrophils, macrophages, and mast cells [65–68]. Deregulation of PGE_2 synthesis leads to a wide range of pathological conditions [69]. In a normal cardiovascular system, PG_{12} acts as a potent vasodilator and TXA_2 as a vasoconstrictor [70, 71]. The presence of both PGI_2 and TXA_2 maintains the normal physiology of the circulatory system [72]. In addition, the myocardium of diabetic and healthy rats does not differ in PG_{12} and PGE_2 [73].

CYP-450-derived eicosanoids 12-HETE and 20-HETE, along with other inflammatory components in diabetic patients, lower the activity of endothelial progenitor cell function. Diabetic vascular complications are associated with

Table 1: Eicosanoid compounds as targets for drug development to control diabetes progression.

Drug	Target	Condition	Consideration	Reference
Celecoxibe	COX-2 inhibitor	Diabetes nephropathy	Female patients received higher dose of PGs vasodilator to maintain blood vessel function than male patients.	[74]
Aspirin	Nonselective COX inhibitor	Diabetes retinopathy	Delay in development of retinal microaneurysms in DR.	[75]
Celecoxibe	COX-2 inhibitor	Diabetes retinopathy	Reduction of vascular leakage.	[76]
Latanoprost	PGF2α agonist	Diabetes retinopathy	Reduces the diameter of dilated retinal arterioles.	[77]
Ketorolac tromethamine	Nonselective COX inhibitor	Diabetes retinopathy	Patients with suspected or visible fibrovascular proliferation demonstrated a reduction in IL-8 and platelet-derived growth factor levels in vitreous humor.	[78]

reduced vascular regenerative potential and nonfunctional endothelial progenitor cell [79].

In sum, imbalanced levels of eicosanoids can induce modification of the microenvironment in the kidneys, eyes, nerves, and cardiovascular system and contribute to the progression of diabetes pathogenesis. Eicosanoid compounds have been studied as targets for drug development to control diabetes progression (Table 1). Thus, we reviewed drugs based on lipid mediators that are involved in diabetes complications.

3. Lipid Mediators in Modulation of Diabetes Complications

When celecoxib, a COX-2 inhibitor, was administered as therapy for diabetic nephropathy in a type 1 diabetes (T1DM) population, COX-2-dependent factors neutralized the angiotensin II effect in the renal microcirculation; further, this effect was greater in women with uncomplicated T1DM than in men [74]. These gender differences could be explained by higher plasma prostanoid found in female animals, an effect that may be estrogen mediated [80–83].

Lower modified levels of PGE_2 relate to changes in the kidney microenvironment and the progression of diabetic nephropathy; thus, PGE_2 and its action are also important targets for drug development [84]. The PGE_2-EP4 pathway contributes to the progression of tubule interstitial fibrosis, and the chronic administration of EP4-agonist in mice, exacerbated inflammation via IL-6, and consequently albuminuria and fibrosis [85]. Additionally, EP4-agonist mediates hyperfiltration in the glomerulus in the early stages of diabetes [86, 87]. Diabetes inflammatory state and chemokine production also increased when mice (T1DM model) were treated with an EP4 agonist [85] and upregulated the development of immune responses Th1 and Th17 [88]. On the other hand, EP receptor antagonists inhibited Th1 and Th17 response [89, 90]. In summary, the activation of the EP4 receptor exacerbates albuminuria levels, inflammation, and fibrosis. COX-2 inhibition reduces albuminuria in renal disease in rats [91]. Recently, using PGE_1 in diabetic nephropathy patients in different disease stages decreased proteinuria and albuminuria [92].

Treating diabetic rats with 50 mg/Kg of aspirin plus 2 mg/Kg of meloxicam (a COX-2 inhibitor) reduced leukocyte adhesion and suppression of the blood-retinal barrier breakdown. This combined dose also reduced retinal ICAM-1 expression, and aspirin alone reduced the expression of C11a, CD11b, and CD18. Together, aspirin and meloxicam reduced the level of TNF-α [93]. Among diabetic patients, 330 mg of aspirin significantly slowed the development of retinal microaneurysms in diabetic retinopathy [75]. Another controlled trial showed that celecoxib reduced vascular leakage in diabetic patients with diabetic retinopathy [76].

Topical administration of nonsteroidal anti-inflammatory drugs (NSAIDs) compared to nontopical administration minimizes systemic exposure to the drug, such that topical NSAIDs can help enhance intraocular penetration. Diabetic patients exhibited elevated plasma IL-8 and elevated vitreous PGE_2 and IL-8 [78, 94]. Exposure to PGE_2 induces IL-8 gene transcription in human T cells [95]. The binding of IL-1β, TNF-α, and IFN-γ also stimulates human retinal pigment epithelial cells to express IL-8 [96]. One study provides direct clinical evidence that topical ocular ketorolac tromethamine (0.45% NSAID) reduces vitreous IL-8 in patients with proliferative diabetic retinopathy [97].

One study found that latanoprost (a $PGF_{2\alpha}$ agonist) used topically significantly reduced dilation of retinal arterioles in type I diabetes patients with diabetic retinopathy, whereas topical diclofenac had no significant effect [77]. In diabetic rats, celecoxib lowered the synthesis of PGE_2 in the retina (a result attributed to selective COX-2 inhibition, since COX-1 inhibitor did not have this effect) [98]. In addition, another COX inhibitor, nepafenac, inhibits increased retinal PG production and leukocyte adhesion in the retinal vessels of diabetes-induced rats [51].

In peripheral arterial diseases, the goal of treatment is to improve symptoms and prevent cardiovascular events [99]. Beraprost sodium is an analogue active PG_{12} with antiplatelet and vasodilating properties [100, 101]. Oral administration of beraprost sodium to diabetic patients improved sensations described as burning/hot, electric, sharp, achy, and tingling

[100]. Beraprost improves symptoms by dilating peripheral vessels and increasing blood flow to the skin [102], and it can also improve painful peripheral neuropathy over a period of 8 weeks [103].

4. Future Perspectives on Eicosanoids

Components of the eicosanoid pathway have a fundamental role in the development of inflammation. As seen in this review, several studies have established that they participate in the progression of diabetes and its complications. Eicosanoids may act as pro- or anti-inflammatory. Currently, PG agonist and COX-1 and/or COX-2 inhibitors are the most promising tools to control diabetes complications, showing good results and promise for the future. Future studies should aim to unveil the function of specific receptors and enzymes acting in more specific targets available only in certain organs, such as the kidneys, eyes, vessels, or nerves.

Conflict of Interests

The authors declare that there is no conflict of interests that would prejudice the impartiality of this scientific work.

Acknowledgments

We thank Sabrina S. Ferreira for assistance with Figure 2. The authors are supported by grant 2010/02272-0 from São Paulo Research Foundation (FAPESP), grant 470523/2013-1 from National Counsel of Technological and Scientific Development (CNPq, Projeto Universal 2013), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Pró-reitoria de Pesquisa da Universidade de São Paulo (PRP/USP, Projeto I and Novos Docentes), Brazil. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 781087, 10 pages
http://dx.doi.org/10.1155/2015/781087

Review Article

Physiological Impact of Abnormal Lipoxin A_4 Production on Cystic Fibrosis Airway Epithelium and Therapeutic Potential

Gerard Higgins,[1,2] Fiona Ringholz,[1,2] Paul Buchanan,[1] Paul McNally,[1] and Valérie Urbach[1,2,3]

[1]*National Children's Research Centre, Crumlin, Dublin 12, Ireland*
[2]*Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland*
[3]*Institut National de la Santé et de la Recherche Médicale, U845, Faculté de Médecine Paris Descartes, Site Necker, 156 rue Vaugirard, 75015 Paris, France*

Correspondence should be addressed to Valérie Urbach; valerie.urbach@gmail.com

Received 20 June 2014; Revised 22 September 2014; Accepted 23 September 2014

Academic Editor: Carlos Artério Sorgi

Lipoxin A_4 has been described as a major signal for the resolution of inflammation and is abnormally produced in the lungs of patients with cystic fibrosis (CF). In CF, the loss of chloride transport caused by the mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel gene results in dehydration, mucus plugging, and reduction of the airway surface liquid layer (ASL) height which favour chronic lung infection and neutrophil based inflammation leading to progressive lung destruction and early death of people with CF. This review highlights the unique ability of LXA_4 to restore airway surface hydration, to stimulate airway epithelial repair, and to antagonise the proinflammatory program of the CF airway, circumventing some of the most difficult aspects of CF pathophysiology. The report points out novel aspects of the cellular mechanism involved in the physiological response to LXA_4, including release of ATP from airway epithelial cell via pannexin channel and subsequent activation of and P2Y11 purinoreceptor. Therefore, inadequate endogenous LXA_4 biosynthesis reported in CF exacerbates the ion transport abnormality and defective mucociliary clearance, in addition to impairing the resolution of inflammation, thus amplifying the vicious circle of airway dehydration, chronic infection, and inflammation.

1. Lipoxin A_4

1.1. Lipoxin A_4 and Eicosanoid Class Switching. Lipoxin A_4 (LXA_4) belongs to a class of newly identified specialised proresolution lipid mediators playing a central role in the resolution of inflammation which results from the sequential production of characteristic eicosanoids in a process termed "class switching" [1, 2]. Prostaglandins are biosynthesized early, initiating the acute inflammatory response. Then Leukotrienes typified by Leukotriene B_4 (LTB_4) play a role in the amplification and propagation of inflammation [1] acting in concert with the peptide Interleukin 8 (IL8) as a potent neutrophil chemoattractant [3, 4]. Both LTB_4 and IL8 are negatively correlated with pulmonary function in CF. LXA_4 is the first eicosanoid expressed in the active resolution phase of inflammation [5] followed by biosynthesis of the Resolvins and Protectins. LTB_4 and LXA_4 are closely related metabolites of arachidonic acid and can be synthesised from a common unstable intermediate [3].

1.2. Lipoxin A_4 Synthesis. LXA_4 is produced by multistep enzymatic process resulting from lipoxygenase (LO) activities in different cell types [6]. Neutrophils [7], eosinophils [8], alveolar macrophages [9], platelets [10], or airway epithelial cells [11] express different LO which act in sequence in LXA_4 biosynthesis [3, 12].

Two main pathways will result in LXA_4 synthesis. One involves lipoxygenation of arachidonic acid by 15-LO in macrophages and epithelial cells. The 5-LO expressed by neutrophils can then utilise the 15(S)-hydroxyeicosatetranoic acid (15S-HETE) released as a substrate to synthesize LXA_4 [7] (Figure 1, blue arrows). Alternatively, platelet 12-LO [10] and macrophage or epithelial 15-LO [13, 14] are each able to transform Leukotriene A_4, released by neutrophils, into

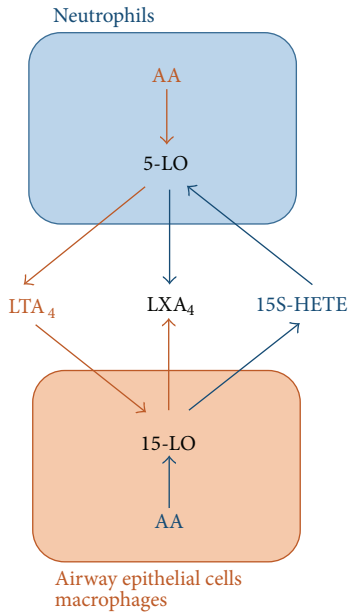

Figure 1: Lipoxin A_4 biosynthesis by trans-cellular cooperation in the airways. The neutrophil donates LTA_4 intermediate formed by the action of 5 lipoxygenase (5-LO) on arachidonic acid (AA) to the acceptor airway epithelial cell or alveolar macrophage whereby 15 lipoxygenase (15-LO) catalyses LXA_4 formation (brown arrows). Airway epithelial cell or alveolar macrophage 15-LO activity catalyses the conversion of AA to 15S-HETE which is donated to the acceptor neutrophil and converted to LXA_4 by 5-LO catalysis (blue arrows).

LXA_4 (Figure 1, brown arrows). The activity of 15-LO promotes LXA_4 biosynthesis and blocks leukotriene biosynthesis, both as a result of 15-LO products competing for flux at the 5-LO level and by diversion of the intermediate Leukotriene A_4 away from LTB_4 towards LXA_4 biosynthesis [1, 11, 15].

1.3. Lipoxin A_4 Anti-Inflammatory Actions. The anti-inflammatory action of LXA_4 is mainly mediated by the formyl-peptide receptor 2 (FPR2) which is one member of a subgroup of receptors linked to inhibitory G-proteins, also called ALX [16, 17]. FPR2 receptor activation by specific agonists results in transient Ca^{2+} flux, phosphorylation of extracellular signal regulated kinases (ERK), and chemotaxis [18]. The molecular and pharmacological characterization of FPR2 receptor have been previously reviewed [19, 20]. Briefly, the seventh transmembrane domain of the FPR2 receptor is essential for LXA_4 recognition, whereas the additional regions of the receptor (e.g., extracellular loops) are required for high affinity binding of the peptide ligands [17, 19, 20]. LXA_4 also interacts directly with the cysLTI receptor to transduce signals that prevent the proinflammatory response and contributes to the active resolution of inflammation [18, 21].

LXA_4 inhibits neutrophil effector functions [5] and in particular inhibits LTB_4 induced neutrophil transmigration [22–24]. LXA_4 suppresses IL8 production by leukocytes and bronchial epithelial cells including airway epithelial cells from patients with cystic fibrosis [25–28]. Mice treated with analogues of LXA_4 and subsequently challenged with *P. aeruginosa* contained the bacterial challenge more effectively [29]. LXA_4 affects leukocytes in a cell type specific manner, inhibiting the activation of polymorphonuclears (PMNs) and eosinophils whilst activating monocytes and macrophages. PMN recruitment is a multistep process that involves chemotaxis, adhesion, and transmigration. In *in vitro* models LXA_4, LXB_4, and ATLS inhibit PMN chemotaxis in response to the chemoattractant LTB_4 and inhibit eosinophil responses to platelet activating factor. Stimulation of macrophages with LXA_4 significantly enhances phagocytosis of apoptotic PMN, suggesting that LXA_4 can promote the clearance of apoptotic leukocytes by macrophages at an inflammatory site [30, 31].

2. Cystic Fibrosis

2.1. Cystic Fibrosis Disease and the CF Gene. Cystic fibrosis (CF) is the most common lethal genetic disorder in Caucasians caused by a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The disease was first characterised in 1938 by Andersen who described the cystic fibrosis of the pancreas and correlated it with the lung and intestinal disease that occurs in CF [32]. In 1953, the observation of excessive salt loss in the sweat of CF patients was noted; however, it was not until 1983 that it was first shown that sufferers of CF displayed abnormal chloride transport. This discovery was not sufficient for the identification of the defective protein in CF patients. In 1985 polymorphic markers associated with the disease were identified and finally the CFTR gene was identified [33–35].

The CFTR protein is principally expressed in the apical membranes of epithelia where it acts as an anion channel providing a pathway for Cl^- and bicarbonate (HCO_3^-) movement, controlling the rate of fluid flow, and also regulating the function of other ion channels and transporters in epithelial cells [36, 37]. A number of different CFTR mutations have been identified that lead to differing outcomes in terms of protein synthesis, trafficking, regulation, and CFTR levels within the cell [38, 39]. CFTR is abundantly expressed in epithelial cell membranes and, as such, CF disease particularly affects epithelial sites: the submucosal glands, airway surface epithelium [40], pancreatic ductal epithelium, the epithelium of the crypts of Lieberkuhn throughout the gastrointestinal tract [41], the epithelium of sweat glands [42], the epithelium of the developing genital ducts, adult epididymis and vas deferens, and the cervical and the uterine epithelial surfaces [43, 44]. However, there are exceptions among epithelial tissues where CF related dysfunction is not prominent, such as kidney collecting ducts, the epithelium of Burners gland, and the submucosal glands of the duodenum [44]. The major clinical features of CF are chronic pulmonary disease, exocrine pancreatic insufficiency, and male infertility. CF lung disease reflects the failure of airway defence against chronic bacterial infection, leading to an aggravated immune response, bronchial epithelial remodelling, and ultimately lung destruction. The progressive lung destruction is the main cause of morbidity and mortality in CF [45, 46]. Whilst it was initially believed that the pulmonary

complaints associated with CF were directly related to the CFTR dysfunction in epithelial cells, it is now recognised that other cell types including neutrophils [47, 48], macrophages [49, 50], and dendritic cells [51] are directly affected by the absence or dysfunctional CFTR.

2.2. Abnormal Production of Lipoxin A_4 in Cystic Fibrosis. In addition to CFTR dysfunction, other abnormalities have been described in chronically inflamed and infected CF airways, including intrinsic proinflammatory properties, amplified inflammatory responses to infections, and reduced bacterial clearance. More specifically, the levels of LXA_4 have been reported to be decreased in CF, like in other chronic airway inflammatory diseases such as asthma [29, 52–55]. A significant suppression in LXA_4/neutrophil ratios in bronchoalveolar lavages (BAL) fluid of patients with CF compared with pulmonary inflammatory controls was reported [29, 56]. Furthermore, in paediatric CF BAL even in the absence of infection, the ratio of LXA_4 to LTB_4 is depressed and this correlates with a significant lower level of 15 LO-2 transcripts in CF BAL [2]. A decreased proportion of proresolving compounds (LXA_4) compared to proinflammatory (LTB_4) is associated with decreased lung function parameters [57]. In addition, *in vitro* studies support a role for CFTR in LXA_4 production. The inhibition of CFTR reduces LXA_4 synthesis by 50% during platelets/PMN coincubation by inhibiting the lipoxin synthase activity of platelets 12-LO. This correlated with the observation that platelets from patients with CF generated 40% less LXA_4 compared to healthy subjects [58]. The decreased LXA_4 production in CF provides a mechanistic explanation of the failure to actively resolve acute airway inflammation seen in these patients.

3. Regulation of Ion Transport and Airway Surface Liquid Layer in Cystic Fibrosis

3.1. Abnormal Ion and Fluid Transport in Cystic Fibrosis. The lung must continually defend itself against bacteria that deposit on the airway surfaces during normal tidal breathing. Mucus clearance is a primary form of pulmonary defence and the efficiency of mucociliary clearance in large part depends upon the volume of the airway surface liquid layer (ASL). The ASL allows for mucus containing foreign bodies to be transported away from the lung to the oropharynx where it is either expelled from the body or swallowed and destroyed by the gut. The ASL provides a low viscosity solution allowing free ciliary beat and mucus transport [59]. The normal hydration of the airway surface is maintained (in the highly water permeable airway epithelium) by active ion-transport controlling the quantity of salt (NaCl) delivered to airway surfaces, with water following passively by osmosis [60]. The NaCl concentration of the airway surface liquid is tightly regulated in normal airway epithelia by the epithelial sodium channel (ENaC) mediated Na^+ absorption and Cl^- secretion. Cl^- is secreted by epithelial cells via the apical CFTR Cl^- channel and calcium activated Cl^- channels, with Cl^- entering the cell through the Na^+–K^+–$2Cl^-$ cotransporter located in the basolateral membrane. Regulation of Cl^- secretion determines the net transport of ions across the epithelium and hence the mass of salt on the epithelial surfaces. CFTR was also found to regulate ENaC suggesting that CFTR acted both as a Cl^- channel and as a regulator of other ion transport processes. In CF, mutations of the CFTR gene result in defective Cl^- secretion and Na^+ hyperabsorption by airway epithelia [61, 62]. Studies in CF airway epithelium cultures, transgenic mice, and people with CF suggest that the initiating event in CF airway disease is a reduced ASL volume resulting from dehydration. This dehydration leads to reduced mucus clearance, adhesion of mucus to airway surfaces, and chronic bacterial infection of the lung (Figure 2). The chronic bacterial infection leads to an aggravated immune response, bronchial epithelial remodelling, and ultimately lung destruction [59, 63–70].

3.2. Lipoxin A_4 Restores Fluid Transport in Cystic Fibrosis. One of the greatest challenges of fundamental research into reversing the CF defect in the lung has been to design a strategy to overcome the absence of functional CFTR by stimulating chloride secretion via alternative pathways, thus restoring airway hydration and mucociliary clearance. This can be achieved via the stimulation of calcium activated Cl^- by agents that raise the intracellular concentration of calcium. Yet, this strategy has been plagued by the side effects of the amplification of the calcium-dependent proinflammatory response resulting in undesirable activation of NFκB. In addition to its anti-inflammatory properties, LXA_4 stimulates a rapid and transient intracellular Ca^{2+} increase in normal and CF bronchial epithelial cells expressing the FPR2 receptor [71, 72]. This intracellular calcium signal is mainly due to calcium mobilisation from intracellular calcium stores in non-CF airway epithelial cells and due to calcium entry and intracellular calcium release in CF airway epithelial cells. In both, non-CF and CF bronchial epithelia, LXA_4 stimulates whole-cell Cl^- currents which are inhibited by NPPB (calcium-activated Cl^- channel inhibitor) and BAPTA-AM (chelator of intracellular Ca^{2+}) but not by CFTRinh-172 (CFTR inhibitor) [71, 72]. Furthermore, in models of fully differentiated bronchial epithelia derived from primary culture of bronchial brushings from patients with CF and cultured under air-liquid interface, LXA_4's effects on ion transport result in an increase of the airway surface liquid (ASL) layer height. LXA_4 exerts this effect on the ASL dynamics via the FPR2 receptor. The sustained increase in ASL height induced by LXA_4 in non-CF and CF bronchial epithelia results from inhibition of amiloride-sensitive Na^+ absorption and stimulation of an intracellular calcium signal and Ca^{2+}-activated Cl^- secretion independent from CFTR [72, 73]. LXA_4 thus restores Cl^- secretion and normal ASL height both central to the pathophysiology of CF airway disease, highlighting a role for LXA_4 in the restoration of normal innate immune defence (Figure 2).

4. Nucleotides and CF Airway Disease

4.1. Regulation of ASL and Mucociliary Clearance by Nucleotides. Mason et al. first proposed that extracellular ATP regulates ion transport rates when added to either the apical

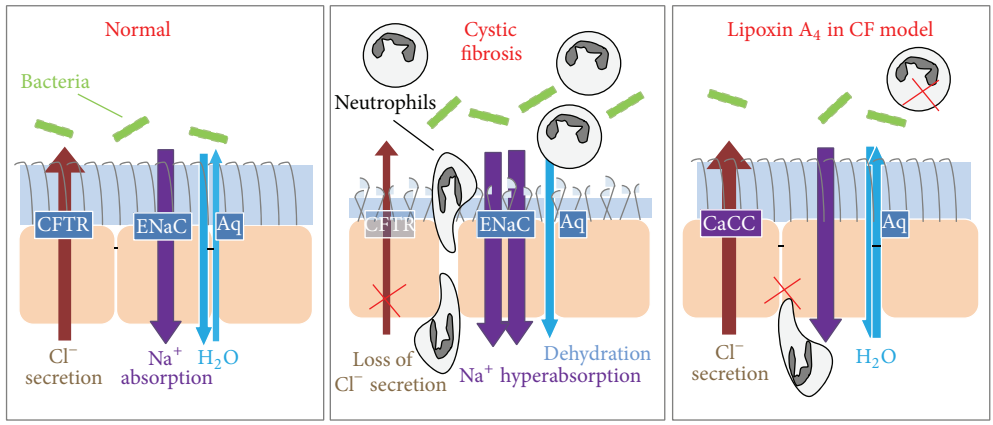

FIGURE 2: In normal airways the airway surface liquid layer (ASL) provides an adequate mucociliary clearance which is maintained by a combination of Cl^- secretion through the cystic fibrosis transmembrane conductance regulator (CFTR), Na^+ absorption via the epithelial sodium channel (ENaC), and water transport through a paracellular pathway and membrane bound aquaporins (Aq). In CF, a defective CFTR leads to loss of Cl^- secretion and Na^+ hyperabsorption. The concomitant dehydration of the airway lumen favours bacterial infection and inflammation (mainly neutrophilic). LXA_4 mediates an increase in ASL height and restores it to normal levels in CF bronchial epithelium. LXA_4 also increase tight junction formation, reestablishing the epithelial barrier function. Taken together this work provides evidence for LXA_4 as potentially a new therapy for CF patients.

or basolateral surface of human airway epithelium and found that these effects appear to be mediated by cell surface receptors that respond to ATP by regulating ion transport rates through the release of Ca^{2+} from internal stores and extracellular Ca^{2+} influx [74]. As agonists were being screened to restore Cl^- and fluid secretion in CF airway epithelium, nucleotide agonists emerged quickly as stimulants of Cl^- and fluid secretion independent of CFTR. Knowles et al. showed that extracellular nucleotides stimulated Cl^- secretion in CF patients. Purinergic agonists in addition to ATP such as UTP, UDP, and ADP also had the power to stimulate Cl^- secretion in CF and non-CF airway epithelial models [75]. In addition, adenosine receptors can also stimulate Cl^- secretion in airway epithelial cells by activating the cAMP/PKA signal transduction pathway and eventually CFTR [76, 77]. ATP signalling through purinergic P2Y receptors is effective in airway epithelia in inhibiting ENaC activity and initiating Ca^{2+}-activated Cl^- secretion [78]. All functionally defined P2Y receptors are able to couple through the IP_3 pathway consisting of activation of PLC increase in inositol phosphates and mobilization of Ca^{2+} from intracellular stores. In addition and secondary to the activation of the PLC, multiple signal transduction pathways including PKC, phospholipase A_2, Ca^{2+} sensitive ion channels, and formation of endothelium derived relaxing factors have been shown to be involved in the responses to activation of native P2Y-receptors. Another function of the P2Y receptors is the activation of ciliary beat frequency. In hydrated airways, the rate of mucociliary clearance is determined by ciliary beat frequency and nonsaturating concentrations of ATP generates alternating Ca^{2+} signals in ciliated cells which in turn increases ciliary beat frequency [79, 80].

Pharmacological data has shown that the P2Y11 receptor is preferentially activated by ATP and is uniquely coupled to both the phosphoinositide and the cAMP pathways [81]. Evidence is available that ATP and ADP, two physiologic nucleotides that can be released into the extracellular space, are able to raise cAMP levels in native cells via activation of P2Y11 receptors. Those results provide a mechanism in addition to activation of P2Y2 or adenosine receptors, by which exogenous or endogenously related nucleotides can increase cellular levels of this important cyclic nucleotide. Given the evidence that a number of types of cells both release ATP and possess P2Y11 receptor, then nucleotide mediated activation of P2Y11 receptors provides a means for autocrine regulation of epithelial and other cell types. Activation of the P2Y11 receptor in different cell types has a number of different outcomes. For example, the P2Y11 receptor mediates the inhibition of neutrophil apoptosis, impaired endothelial cell proliferation or regulation of secretory function of pancreatic ductal cells by ATP [82–84].

4.2. Nucleotides Release by Pannexin Channel. The complex cellular composition of the airways that is ciliated cells and mucin-secretory goblet cells suggests that several mechanisms and pathways are involved in the release of nucleotides into the airways. Two general mechanisms for the release of ATP from cells have been proposed as vesicular release and channel-mediated release. While vesicular release of ATP is well documented, ATP release can also occur in the absence of vesicules. For example, human erythrocyte which is devoid of cytoplasmic vesicle can release ATP in low oxygen content or in response to shear stress [85]. Pannexins belong to the family of connexin channels that have been proposed as diffusion pathways for ATP release under various experimental conditions. The Pannexins primarily form oligomeric structures embedded in a single plasma membrane that when open provide a conduction pathway between cytosol and extracellular space. They are mechanosensitive and are highly permeable to ATP [86]. Exposure of the alveolar A549 cells to

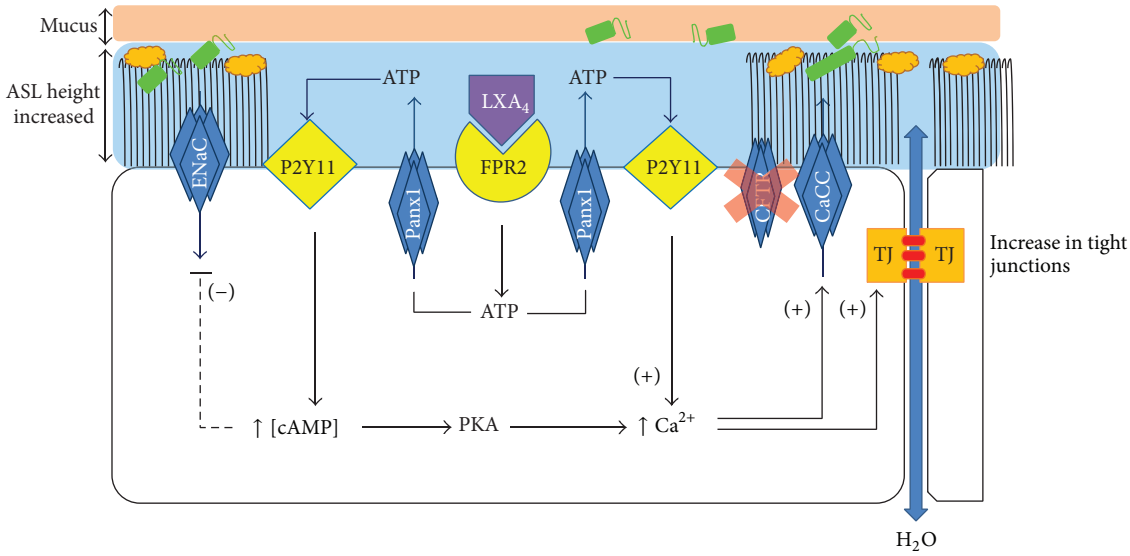

Figure 3: Lipoxin A_4 enhances epithelial barrier integrity by stimulating an increase in airway surface liquid (ASL) layer height, epithelial repair, and tight junction formation. Stimulation of the FPR2 receptor by LXA_4 induces an apical ATP release through the pannexin (Panx1) channel activating a purinoreceptor pathway. Activation of P2Y11 receptors stimulates chloride secretion out of the cell by calcium activated chloride channels (CaCC) and inhibition of sodium absorption by amiloride sensitive epithelial sodium channels (ENaC) which result in a restored ASL height in CF bronchial epithelial cells. The calcium signal induced by P2Y11 activation also stimulates epithelial repair and tight junction formation. Taken together, the physiological effects induced by LXA_4 have the potential to delay the invasion of bronchial epithelial cells by bacteria (green and orange structures).

thrombin resulted in a strong ATP release response that was inhibited by the nonselective blockers of pannexin channels suggesting that ATP release from thrombin-stimulated lung epithelial cells occurs through pannexin channels [87]. A study by Ransford et al. 2009 showed ATP release induced by hypotonic shock of human bronchial epithelial cells was inhibited after silencing pannexin-1 (Panx1) via shRNA [88]. The large pores of Panx1 (the most studied pannexin channel) are permeable to ions, second messengers, and neurotransmitters such as ATP, IP_3, and amino acids. Panx1 is also implicated in secretion of arachidonic acid and its metabolites and it is now widely regarded that Panx1 membrane channels are also involved in the extracellular mode of wave propagation. Panx1 channels open in response to mechanical stress or other stimuli such as depolarization and release ATP to the extracellular medium. ATP binding to purinergic receptors triggers an increase of cytoplasmic Ca^{2+} via the IP_3 pathway. The Ca^{2+} increase is not restricted to the same cell but also includes cells within diffusion distance for the released ATP also stimulating cells that are coupled to the stimulated cell by gap junction channels permitting the flux of IP_3.

The increase in Ca^{2+} can activate Panx1 channels and subsequent release of ATP provides a new source for extracellular ATP to reach more distant cells [86]. The application of micromolar concentrations of Ca^{2+} to the cytoplasmic side of Panx1 channels in excised membrane patches activated the channels at negative membrane potentials where the channels are normally closed [86].

4.3. Lipoxin A_4 Increases the Airway Surface Liquid Height via P2Y11 Activation. The mechanism by which LXA_4 stimulates Ca^{2+}-activated Cl^- secretion and ASL height increase has been elucidated. Higgins et al. reported that LXA_4 induces an apical ATP release from non-CF and CF airway epithelial cell lines and CF primary cultures. This ATP release induced by LXA_4 is completely inhibited by antagonists of the FPR2 receptor and Panx1 channels suggesting a major role of Panx1 in this effect. Furthermore, LXA_4 induces an increase in intracellular cAMP and calcium, which are abolished by the selective inhibition of the P2RY11 purinoreceptor. Panx1 and ATP hydrolysis inhibition and P2RY11 purinoreceptor knockdown all abolish the increase of ASL height induced by LXA_4. Inhibition of the A_2b adenosine receptor does not affect the ASL height increase induced by LXA_4, whereas the PKA inhibitor partially inhibits this response. Taken together this report provides evidence for a novel role of LXA_4 in stimulating apical ATP secretion via Panx1 channel and subsequent P2RY11 purinoreceptor activation in airway epithelial cells leading to an ASL height increase (Figure 3).

5. Epithelial Repair in CF Airway

5.1. Altered Epithelial Repair in CF. In CF, recurrent infections and inflammatory insults result in damage to the airways and trigger the repair process [89]. Epithelial repair initially involves cell migration and cell proliferation to repopulate the injured area [90–92]. This process is then followed by differentiation of the epithelium [93]. Recent research suggests that epithelial repair as well as differentiation of the CF airway epithelium is downregulated or delayed [94–98]. More specifically, cell migration and proliferation both appear to be reduced during repair of CF bronchial

epithelial cells compared to non-CF cells [99]. This delay in repair of the CF epithelium renders the lung more susceptible to ongoing bacterial infection and thus may trigger more epithelial damage [100].

5.2. Lipoxin A_4 Regulates Airway Epithelial Integrity in CF Airway Epithelium. The lipid mediator LXA_4 triggers epithelial cell migration and proliferation and thus plays a role in repair of epithelia including bronchial epithelium from patients with CF [22, 99, 101–104]. The effects of LXA_4 in stimulating cell proliferation, cell migration, and wound repair are mediated by the apical ATP release and P2Y11 activation [105]. Stimulation of P2Y11 purinoreceptors induces calcium release and ERK phosphorylation, both of which play a key role in initiating cell proliferation and migration [106–113]. Furthermore, consistent with the role of potassium channels in two key processes of repair, migration, and proliferation in numerous cell types, the responses to LXA_4 on the repair process are mediated by the downstream activation of K_{ATP} potassium channels [96, 97, 99, 114–117]. Additionally, LXA_4 enhances airway epithelial tight junction formation which is a main factor of epithelial barrier integrity. LXA_4 stimulates ZO-1, claudin-1, and occludin expression and trafficking at the apical membrane resulting in enhanced transepithelial electrical resistance in human airway epithelia [118] (Figure 2). Taken together, these effects of LXA_4 on airway epithelial structure suggest the abnormal levels of LXA_4 in CF airways may account for the reduced capacity for epithelial repair in CF.

6. Treatments of CF Airway Disease

6.1. Current Treatments and Opportunities. There is currently no treatment available that fully corrects the biochemical abnormality in CF and leads to a cessation of the typical pathobiology seen in the condition. Therapies to date have been centred on slowing the decline in pulmonary function over time to prolong survival. Medication is predominantly used to optimise nutrition (pancreatic enzymes, fat soluble vitamin supplementation), treat infection (oral, inhaled, and intravenous antibiotics), and facilitate effective mucociliary clearance (DNAse, hypertonic saline). Several anti-inflammatory approaches have been examined in CF; however, the ideal anti-inflammatory drug is not yet available [119]. A recent systematic review of the risks and benefits of inhaled corticosteroids in CF, examining evidence from 13 trials, concluded that there is insufficient evidence to establish whether they are beneficial in CF while it is established that ICS use can have adverse effects [120]. A systematic review of the efficacy of nonsteroidal anti-inflammatory drugs in CF concluded that treatment with high-dose ibuprofen was associated with a significantly lower annual rate of decline in lung function (especially in children); however, the adoption of ibuprofen into therapy has not been universally accepted [121]. Correcting the imbalance in fatty acid metabolism described in CF by supplementation of Docosahexaenoic Acid may be helpful, and efforts are ongoing to evaluate the potential therapeutic benefit [122].

Two promising avenues of therapy have recently emerged: small molecule correctors and gene therapy. The flagship small molecule corrector has been Ivacaftor (VX-770). This compound facilitates gating of defective CFTR where the cause of CFTR dysfunction is a gating mutation—predominantly G551D. This has been remarkably clinically successful but can be taken by only approximately 5% of patients worldwide [123, 124]. The manufacturers of Ivacaftor, Vertex Pharmaceuticals, are currently developing correctors for the commonest mutation Phe508del. Phase 2 trials of this compound have been shown to lead to positive changes in CFTR function, but not to the same degree as VX-770 [125]. Gene therapy was considered an obvious target for disease modifying treatment after the discovery of the CFTR gene; however initial attempts at this approach were unsuccessful, prompting a comprehensive review of the process of selection of endpoints, vectors, and delivery modes. A consortium in the UK has developed a comprehensive approach in this regard and will report shortly on multidose trials of gene therapy in individuals with CF [126]. A treatment approach capable of effectively preventing lung damage and decline in pulmonary function is currently absent despite the obvious hope relating to new developments.

For now, we continue to search for new and effective therapies to slow or prevent the decline in pulmonary function in CF.

6.2. Therapeutic Potential for LXA_4 in the Treatment of CF Airway Disease. A variety of airway clearance therapies have been developed for patients with CF [127, 128]. Thus identification of agents, particularly endogenous biologicals that stimulate non-CFTR Cl^- secretory pathways and promote ASL height recovery while providing anti-inflammatory effects are likely to be of therapeutic benefit in improving mucociliary clearance in patients with CF. The effect of LXA_4 inhalation has been evaluated in a pilot study of eight asthmatic and healthy adult subjects. The challenge was tolerated, had no adverse effect on pulse or blood pressure, and demonstrated favourable effects on specific airway conductance [129].

In conclusion, the discovery of the multiple impacts of LXA_4 in restoring bronchial epithelium ion transport, in enhancing ASL height, in restoring epithelial barrier function, and in reducing inflammation might provide significant advance in treatment of the CF airway disease (Figure 3).

Conflict of Interests

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

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 848762, 7 pages
http://dx.doi.org/10.1155/2015/848762

Review Article

Multifaceted Roles of Cysteinyl Leukotrienes in Eliciting Eosinophil Granule Protein Secretion

Renata Baptista-dos-Reis, Valdirene S. Muniz, and Josiane S. Neves

Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Centro de Ciências da Saúde (CCS), 373 Carlos Chagas Filho Avenue, Room F 14, 1st Floor, Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil

Correspondence should be addressed to Josiane S. Neves; josiane.sabbadini.neves@gmail.com

Received 12 June 2014; Accepted 9 October 2014

Academic Editor: Ruxana Sadikot

Cysteinyl leukotrienes (cysLTs) are cell membrane-impermeant lipid mediators that play major roles in the pathogenesis of eosinophilic inflammation and are recognized to act via at least 2 receptors, namely, $cysLT_1$ receptor ($cysLT_1R$) and $cysLT_2$ receptor ($cysLT_2R$). Eosinophils, which are granulocytes classically associated with host defense against parasitic helminthes and allergic conditions, are distinguished from leukocytes by their dominant population of cytoplasmic crystalloid (also termed secretory, specific, or secondary) granules that contain robust stores of diverse preformed proteins. Human eosinophils are the main source of cysLTs and are recognized to express both cysLTs receptors (cysLTRs) on their surface, at the plasma membrane. More recently, we identified the expression of cysLTRs in eosinophil granule membranes and demonstrated that cysLTs, acting via their granule membrane-expressed receptors, elicit secretion from cell-free human eosinophil granules. Herein, we review the multifaceted roles of cysLTs in eliciting eosinophil granule protein secretion. We discuss the intracrine and autocrine/paracrine secretory responses evoked by cysLTs in eosinophils and in cell-free extracellular eosinophil crystalloid granules. We also discuss the importance of this finding in eosinophil immunobiology and speculate on its potential role(s) in eosinophilic diseases.

1. Introduction

Cysteinyl leukotrienes (cysLTs) constitute an important class of potent proinflammatory mediators. These leukotrienes are synthesized from membrane-derived arachidonic acid via the 5-lipoxygenase (5-LO) pathway in concert with the 5-LO-activating protein (FLAP), forming LTA_4, which is converted into LTC_4 by the action of LTC_4 synthase [1]. The production of LTC_4 within cells may occur at perinuclear membranes or in cytoplasmic lipid bodies, which are cytoplasmic organelles rich in lipids. The formation of these bodies is highly regulated, and they have functions in eicosanoid production and storage of inflammatory proteins [2]. Intracellular LTC_4 is actively transported extracellularly, where it is enzymatically sequentially converted to LTD_4 and then to LTE_4 [1]. CysLTs are cell membrane-impermeant and are recognized to mediate their actions by engaging at least two heptahelical G protein-coupled receptors (GPCRs), designated $cysLT_1$ receptor ($cysLT_1R$) and $cysLT_2$ receptor ($cysLT_2R$), which are expressed on the cell surface, at the plasma membrane [1, 3]. The rank orders of the affinities of cysLTs for human $cysLT_1R$ and $cysLT_2R$, based on research in transfected cells, are $LTD_4 \gg LTC_4 = LTE_4$ and $LTC_4 = LTD_4 > LTE_4$, respectively [4, 5]. However, various findings suggest the existence of another not yet cloned cysLT receptor (cysLTR), since numerous cysLTs' biological actions are not well explained by their affinities to the known cysLTRs [6–11]. For instance, experiments in animal models and human studies have revealed that LTE_4, considered the weakest cysLTRs agonist, has unique characteristics that cannot be explained by current knowledge of $cysLT_1R$ and $cysLT_2R$ [9–11]. Moreover, an additional receptor sensitive to LTE_4, the purinergic P2Y12 receptor (P2Y12R), has been identified by *in silico* and *in vivo* methods [12, 13]. In contrast, other studies have suggested that LTE_4, as well as other cysLTs, does not activate intracellular signaling by acting through P2Y12R and that another LTE_4-specific receptor has yet to be identified [14]. In fact, homo- and heterodimerization of

cysLTRs and purinergic receptors have been widely suggested [15, 16]. Whether the 3 candidate cysLTRs function or interact as homo- or heterodimers is not known. More studies are still needed to better clarify this point.

Human eosinophils are major sources of cysLTs and express both $cysLT_1R$ and $cysLT_2R$ on their plasma membranes [1, 17]. CysLTs and their receptors have critical roles in allergic diseases and represent important therapeutic targets for the control of asthma and other pathophysiological conditions [15, 18]. Within eosinophils, synthesis of LTC_4 (but not extracellularly formed LTD_4 or LTE_4) occurs at perinuclear membranes and in cytoplasmic lipid bodies [17]. Mature eosinophils contain a single population of secondary (or specific or crystalloid) granules that are ultrastructurally characterized as membrane-bound organelles containing a crystalloid core surrounded by a matrix. Based on diverse electron microscopy and subcellular fractionation studies, it is now recognized that human eosinophils synthesize and store cationic proteins, such as eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), eosinophil granule major basic protein 1 (MBP-1), enzymes, growth factors, chemokines (such as RANTES and eotaxin), and over 36 cytokines (including Th1 and Th2 cytokines) that are selectively secreted in response to a range of stimuli and agonists [19–26]. Mechanisms for differentially mobilizing these granule-stored proteins for their extracellular release may enable eosinophils to selectively and rapidly influence various immune, inflammatory, and other responses. The secretion of granule contents from intact eosinophils primarily occurs by a mechanism termed piecemeal degranulation (PMD). This is a process whereby granule contents are selectively mobilized into spherical and tubular vesicles that need to disengage from the granules, transit through the cytoplasm, and fuse with the plasma membrane to release their specific granule-derived protein cargo at the cell surface [21, 23, 27]. Another mechanism of human eosinophil "degranulation" is associated with cytolysis. Following lysis of an eosinophil, with loss of its plasma membrane integrity, intact, cell-free, membrane-bound granules are released and deposited extracellularly. Although PMD is considered to be the predominant mechanism underlying eosinophil degranulation and secretion, cytolysis has been recognized as a common mechanism for cell-free eosinophil granule release and deposition in tissues in eosinophilic diseases [28–31]. Compound exocytosis, whereby the entire granule contents are released extracellularly following fusion of the granules with the plasma membrane, occurs when eosinophils interact with large targets, such as helminthic parasites. However, this process is not usually observed *in vivo*.

Given that the selective release of cytokines can provide a mean for eosinophils to rapidly influence adjacent cells in normal or inflamed tissues, investigation of the mechanisms involved in the selective mobilization and vesicle-mediated secretion of specific cytokines, including IL-4 and other preformed cytokines, is extremely relevant. Several studies have provided new insights into the signal transduction processes that contribute to the selective mobilization and release of specific eosinophil granule-derived cytokines and chemokines [22, 32–36]. Several of these studies have identified the intracellular expression of cysLTRs and cysLT production as important inflammatory mediators eliciting the secretion of specific cytokines from eosinophils and from cell-free extracellular eosinophil granules [32–34, 36]. It appears increasingly likely that eicosanoids synthesized within cells, including eosinophils, may have other important intracrine roles in regulating cell functions, in addition to their more recognized autocrine/paracrine activities in inflammation.

2. CysLTs Are Intracrine Signals Regulating Eosinophils' IL-4 Secretion by Piecemeal Degranulation

In eosinophils, it is noteworthy that in addition to their recognized activities as autocrine/paracrine mediators, eicosanoids such as cysLTs are now also recognized to display intracrine effects. The cysLTs, LTC_4, and their extracellular derivatives, LTD_4 and LTE_4, are recognized as paracrine mediators pertinent to asthma and allergic diseases based on their receptor-mediated capabilities to elicit bronchoconstriction, mucous hypersecretion, bronchial hyperresponsiveness, increased microvascular permeability, and additional eosinophil infiltration [1, 15, 18, 37]. Eosinophils are major sources of cysLTs [17] and are the principal LTC_4 synthase-expressing cells in bronchial mucosa biopsy specimens from asthmatic subjects, as well as being recognized to express both cysLTRs [1, 17]. Thus, cysLTs are also important autocrine regulators of eosinophil function. Indeed, a series of reports showed that cysLTs have the ability to affect various eosinophil responses [35, 38–41]. For instance, in eosinophils derived from human cord blood progenitors *in vitro*, it was shown that LTC_4, LTD_4, and LTE_4 induced dose- and time-dependent, vesicular transport-mediated release of preformed IL-4 [38]. Although controversy exists [39], cysLTs also appear to be able to induce the *in vitro* survival of human eosinophils by activation of $cysLT_1R$ [40, 41]. Additionally it was demonstrated that enhanced plasma membrane expression of activation-related CD69 on human eosinophils induced by platelet-activating factor (PAF) and IL-5 is dependent on endogenous eosinophil-derived 5-LO metabolites [35]. Consequently, much interest in understanding the regulation of eicosanoid formation in eosinophils has focused on the mechanisms that regulate eosinophil cysLT formation and release. Interestingly, it was noted that depending on the stimulus, the localized synthesis of LTC_4 may occur at distinct intracellular sites within eosinophils (at the perinuclear membrane and/or in lipid bodies) and may control the role of this mediator as either an intracrine signal-transducing mediator that regulates PMD and cytokine secretion or an autocrine/paracrine element in eosinophilic inflammation [2, 32, 36]. In 2002, Bandeira-Melo and colleagues [32] evaluated whether cysLTs function as intracrine mediators involved in the stimulated release of IL-4 from eosinophils. The authors demonstrated that although eotaxin and RANTES each act via CCR3 to stimulate the secretion of both IL-4 and RANTES from eosinophils, only the release of IL-4 was dependent

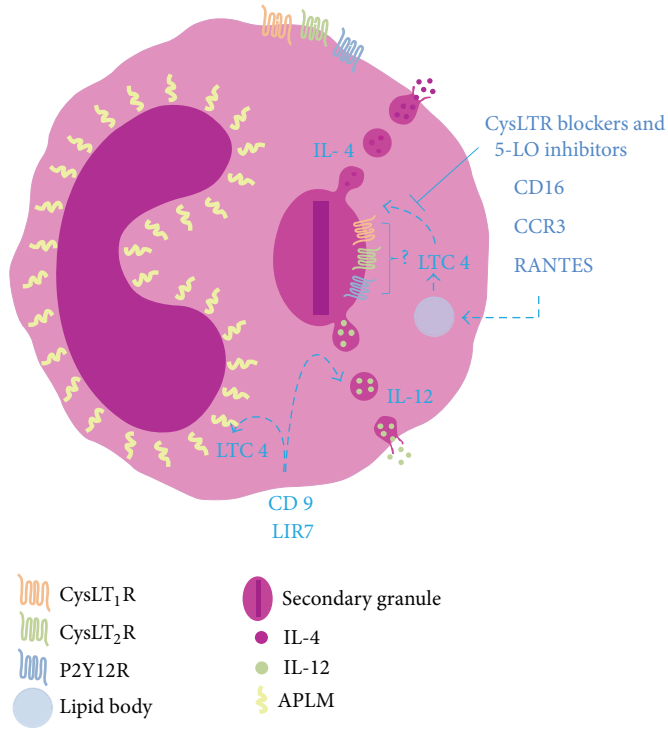

Figure 1: CysLTs are intracrine signals regulating eosinophils' IL-4 secretion by piecemeal degranulation.IL-4 release induced by IL-16, eotaxin, and RANTES is dependent on the intracrine action of lipid body-generated LTC_4. Inhibitors of 5-lypoxigenase (5-LO) and/or cysteinyl leukotriene receptors (cysLTRs) blocked intracellular LTC_4 production and consequently IL-4 release from eosinophils. The cross-linking of LIR7- or CD9-induced perinuclear-generated LTC_4, however IL-12 secretion induced by LIR7 or CD9 is independent of 5-LO metabolites. $CysLT_1R$ = $cysLT_1$ receptor; $cysLT_2R$ = $cysLT_2$ receptor, P2Y12R = purinergic P2Y12 receptor, and APLM = arachidonyl phospholipids and lipoxygenase machinery.

on the activation of 5-LO to form LTC_4 within eosinophils' lipid bodies. Inhibitors of 5-LO blocked IL-16-, eotaxin-, and RANTES-induced IL-4 release, but exogenous LTC_4, LTD_4, and LTE_4 did not elicit IL-4 release. Only after membrane permeabilization were cysLTs enabled to enter eosinophils and stimulate IL-4 but not RANTES release. LTC_4- and LTD_4-elicited IL-4 release was pertussis toxin inhibitable, but inhibitors of the two known GPCRs, $cysLT_1R$ and $cysLT_2R$, did not block LTC_4-elicited IL-4 release. LTC_4 was more potent than LTD_4 was and, at low concentrations, elicited IL-4 release from permeabilized eosinophils, whereas higher concentrations were inhibitory probably due to the high-dose inhibition characteristic of the GPCRs. For intact eosinophils, also as a consequence of high intracellular LTC_4 levels, LTC_4 export inhibitors blocked eotaxin-elicited IL-4 release. Thus, taken together, these data demonstrate that despite being well recognized as an autocrine/paracrine mediator, LTC_4, via an intracellular cysLTR distinct from $cysLT_1R$ and $cysLT_2R$, may also dynamically govern inflammatory responses as an intracrine mediator of eosinophils' PMD-mediated cytokine secretion (Figure 1).

Interestingly, in a different study, Tedla and colleagues showed that the cross-linking of leukocyte immunoglobulin-like receptor 7 (LIR7) and CD9 with immobilized antibodies induced LTC_4 generation at the nuclear envelope and the release of IL-12, but not IL-4, by vesicular transport [36]. Whereas the IL-4 release induced by IL-16 and CCR3-activating chemokines is dependent on the intracrine action of lipid body-generated LTC_4 [32], the IL-12 release induced by the cross-linking of LIR7 does not appear to be regulated by 5-LO metabolites [36, 42]. Pretreatment with two mechanistically distinct inhibitors of 5-LO (AA861 and MK886) blocked IL-16-, eotaxin-, and RANTES-induced LTC_4 production and IL-4 release from eosinophils [32]. In contrast, pretreatment of eosinophils with either AA861 or MK886 did not inhibit the selective release of IL-12 induced by the cross-linking of CD9 or LIR7, indicating that 5-LO does not participate in CD9- or LIR7-driven selective IL-12 release [36, 42]. Moreover, stimulation of permeabilized eosinophils with LTC_4 did not elicit IL-12 release [36]. Overall, intracellular LTC_4 formed in lipid bodies appears to function as an intracrine, and not an extracellular autocrine/paracrine, mediator to regulate the differential secretion of IL-4 induced by IL-16, eotaxin, or RANTES [32]. Meanwhile, the intracellular 5-LO-derived LTC_4 formed at the perinuclear membrane appears not to control the selective IL-12 release induced by the cross-linking of CD9 or LIR7 and may function as an autocrine/paracrine mediator of inflammation [36]. These studies suggest that the capacity of eosinophils to synthesize LTC_4 in lipid bodies may relate

less to paracrine mediator formation and more to intracrine signal-transducing activities pertinent to more local transcriptional or other cellular functions [43] (Figure 1).

Possible intracrine roles for LTC_4 have also been described in other cell types, including vascular and mast cells; however, how LTC_4 acts intracellularly remains to be defined [44–46]. Although eosinophils express the two known cysLTRs, $cysLT_1R$ and $cysLT_2R$ [17, 42], little is known about the intracellular distribution of these receptors in eosinophils. In addition to its conventional plasma membrane expression, $cysLT_1R$ has been immunolocalized to nuclei in colorectal adenocarcinoma cells [47], in a human mast cell line [48] and in vascular smooth muscle cells [46]. The functions of nuclear $cysLT_1R$ are poorly understood. For instance, in one interesting study, Nielsen and colleagues demonstrated that isolated intestinal cell nuclei express $cysLT_1R$ and respond to LTD_4, triggering ERK1/2 signaling [47]. However, whether these nuclear-localized receptors are involved in the cell cycle (for survival or proliferation) is still unknown. In a different study, Eaton and colleagues showed that LPS upregulated the perinuclear expression of $cysLT_1R$ in vascular smooth muscle cells and that LTC_4 stimulation predominantly enhanced nuclear calcium increase and gene transcription [46]. Whether or how exogenous LTC_4 reaches these intracellular $cysLT_1Rs$ is still not defined. Recently, we defined the intracellular expression of cysLT-sensitive receptors in crystalloid granule membranes [34]. These findings might help in identifying novel mechanisms whereby cysLTs can serve as intracrine mediators.

3. Extracellular Eosinophil Granules Express Ligand-Binding Domains for CysLTRs on Their Membranes and Secrete ECP in Response to CysLTs

Intracrine roles for cysLTs are described in the literature, but the mechanisms involved that can explain cysLTs' intracellular actions remain unknown [32, 44, 45]. A description of intracellular cysLTRs expression in human eosinophils was recently provided by our group [34]. In 2010, we reported, for the first time, that the receptors for cysLTs, $cysLT_1R$ and $cysLT_2R$, and the purinergic P2Y12R are expressed on eosinophil granule membranes [34]. We showed that eosinophil granules express amino-terminal, ligand-binding domains for $cysLT_1R$ and $cysLT_2R$ and the P2Y12R on their membranes. We previously observed that certain cytokine and chemokine receptors are richly present on eosinophil granules [22, 33, 49]. These granules, upon extrusion from eosinophils, responded to a stimulating cytokine, interferon-γ, and a chemokine, eotaxin-1 (CCL11), via cognate granule membrane-expressed receptors to activate intragranular signaling pathways that elicit granule protein secretion [33, 50]. Stimulating cell-free eosinophil granules with the agonists LTC_4, LTD_4, and LTE_4 elicited the secretion of ECP, but not eosinophil-derived cytokines or chemokines, from the granules (as detected by cytokine multiplex assays). Montelukast, a recognized inhibitor that principally inhibits $cysLT_1R$, as well as the P2Y12R antagonist MRS 2395, inhibited eosinophil granule ECP secretion after $LTC_4/LTD_4/LTE_4$ stimulation of cell-free eosinophil granules [34] (Figure 2). The capacity of a $cysLT_1R$ inhibitor or a P2Y12R antagonist, such as montelukast and MRS 2395, respectively, to similarly inhibit the secretion elicited by ligands (e.g., LTE_4) not active for $cysLT_1R$ or not classically selective for the receptor (e.g., P2Y12R), suggests functional heterodimerization of $cysLT_1R$ and other receptors (e.g., functional heterodimerization between $cysLT_1R$, $cysLT_2R$, and P2Y12R) expressed on eosinophil granule membranes; whether this is the case remains to be ascertained. In addition, montelukast's potential off-target effects could not be discounted. Notably, the dose response to the three cysLTs varied. LTC_4 and LTE_4 elicited ECP secretion only at lower (subnanomolar) concentrations, which was fully consistent with the high-dose inhibition characteristic of the GPCRs. Intriguingly, LTD_4 elicited ECP secretion at low and high, but not intermediate, concentrations. This dose response suggests the engagement of two receptors sensitive to LTD_4, with the first responding to low LTD_4 levels and then exhibiting higher dose inhibition and the second receptor putatively mediating secretion at higher concentrations of LTD_4. As previously mentioned, oligomerization of leukotriene and purinergic receptors has been widely suggested [15, 16]. However, whether dimerization of receptors is involved in this response remains to be elucidated. These findings highlight the capacity of cysLTRs to stimulate cell-free granule secretory responses. Furthermore, for granules serving as intracellular organelles these data identify novel mechanisms whereby LTC_4 and extracellularly generated LTD_4 and LTE_4 (if these mediators could be, by any chance, internalized by the cell) may serve as intracrine mediators of eosinophil granule-derived secretion. However, there is no evidence that the $cysLT_1R$, $cysLT_2R$, or P2Y12R expressed on granule membranes is involved in the intracrine actions of cysLTs described previously [32]. This phenomenon is not likely, considering that LTC_4- and LTD_4-elicited IL-4 release in permeabilized eosinophils is not blocked by inhibitors of $cysLT_1R$ and $cysLT_2R$ [32].

All of these findings are remarkable because they provide additional information about the capacity of eosinophils to contribute to modulating host and inflammatory responses after eosinophil cytolysis. Cytolytic release of intact eosinophil granules yields extracellular organelles fully capable of ligand-elicited active secretory responses, amplifying the differential secretory properties of eosinophils and likely contributing to the persistence and exacerbation of the inflammatory response.

4. Implications for Eosinophilic Diseases and Eosinophil Immunobiology: Questions for the Future

Intact membrane-bound eosinophil granules have long been recognized to be present extracellularly in tissues and secretions in many human eosinophil-enriched disorders (for review see [49]). The capacity of cell-free human eosinophil granules to act via receptor-mediated responses to cysLTs and

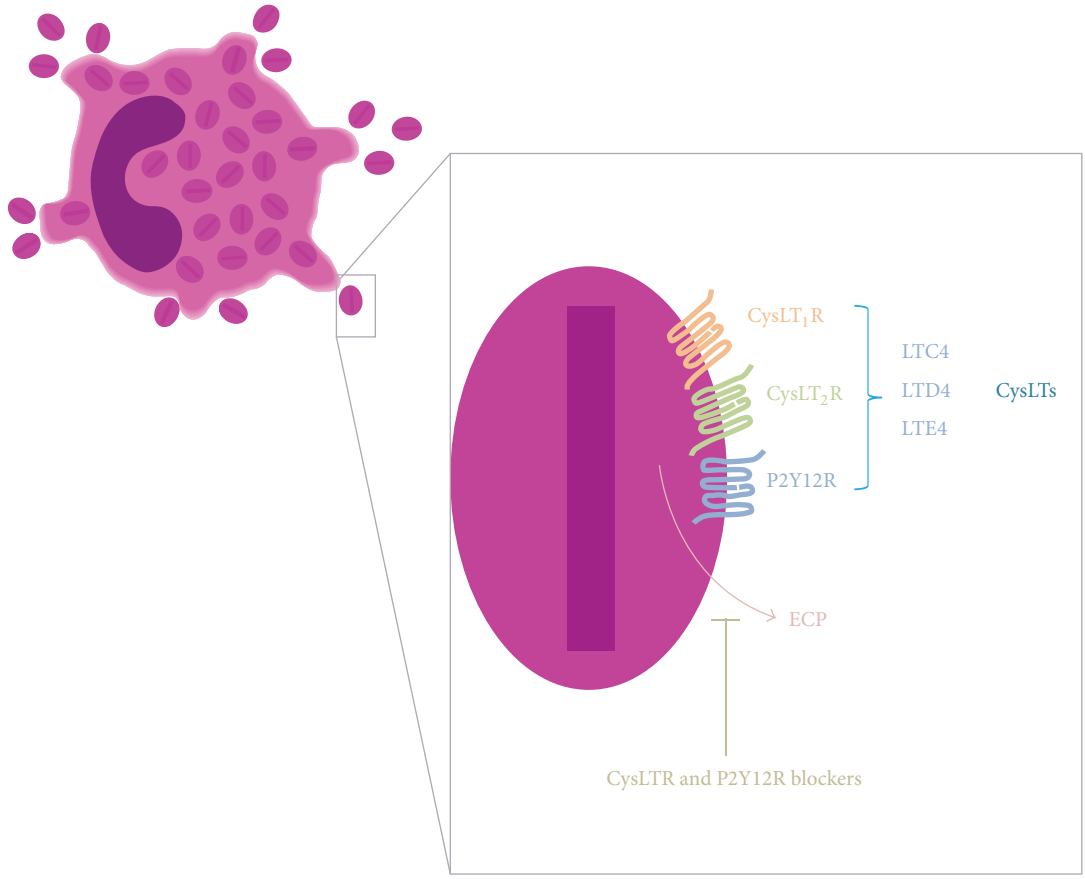

Figure 2: Stimulating cell-free eosinophil granules with the agonists LTC_4, LTD_4, and LTE_4 elicited the secretion of eosinophil cationic protein (ECP) but not eosinophil-derived cytokines or chemokines from the granules. Cysteinyl leukotriene receptor (cysLTR) or P2Y12 receptor (P2Y12R) blockers inhibited ECP secretion after LTC_4/LTD_4/LTE_4 stimulation of cell-free eosinophil granules.

to secrete granule-derived cationic proteins has indicated that cell-free eosinophil granules may be functionally significant in response to these lipid mediators [34], likely contributing to perpetuation of the inflammatory process in an affected organ. As intracrine mediators, the capacity of eicosanoids, such as LTC_4, to be synthesized at 2 discrete sites, perinuclear membranes and lipid bodies, in eosinophils raises questions about the roles of these pools of lipids in the functioning of eosinophils in inflammation. As noted above, cysLTs are well-recognized autocrine/paracrine mediators that are pertinent to eosinophils, asthma, and allergic inflammation; however, how these eicosanoids transit from their intracellular sites of synthesis for their extracellular release, which is requisite for their autocrine/paracrine functions, remains uncertain. This uncertainty is especially true for LTC_4 formed at perinuclear membranes that are not proximate to the cell surface. Furthermore, the logic of using phospholipids resident in perinuclear membranes as a source of substrate for forming secreted eicosanoid mediators remains unclear. Therapeutic agents in current use that inhibit 5-LO or $cysLT_1R$ may have activities broader than simply blocking the paracrine-mediator activities of cysLTs. Furthermore, with greater understanding of the intracellular sites of cysLT synthesis and cysLTR expression and of the functional consequences of such cysLTR-mediated cellular regulation for both intracellular and cell-free granules, newer therapeutic agents may be targeted to regulate specific activities of cysLTs pertinent to asthma and other allergic diseases.

Notably, studies demonstrating that isolated human eosinophil granules could exert extracellular functions as secretion-competent organelles after stimulation with ligands, including chemokines and cysLTs [33, 34], provide highly relevant observations in terms of eosinophil cell biology. Considering functional roles for these intracellular granule membrane-expressed receptors, with their ligand-binding domains displayed on the outer granule membranes, these findings not only support extracellular granule function but also suggest that the granule-expressed receptors potentially serve as intracrine mediators of eosinophil-derived granule secretion. For receptors such as the lipid-mediator receptors ($cysLT_1R$, $cysLT_2R$, and P2Y12R), which are activated by hydrophobic ligands that can be synthesized at the nuclear membrane or in lipid bodies, it is possible to predict roles in intracellular compartments, where they would be accessible to their ligands. For chemokine/cytokine receptors, it is also feasible that the ligands themselves are active intracellularly after their biosynthesis and/or based on specific cell uptake and internalization mechanisms.

Current investigations are only beginning to explore the functional biology and responses of eosinophil granules. Most notably, how specific granule-derived proteins are selectively mobilized for secretion from either isolated eosinophil granules or intracellular granules by PMD within intact eosinophils is yet to be ascertained. The molecular mechanisms that regulate eosinophil granule protein mobilization and secretion continue to be intriguing and requiring further delineation.

Conflict of Interests

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

Acknowledgments

CNPq/Brazil (to Valdirene S. Muniz, Renata Baptista-dos-Reis, and Josiane S. Neves) and FAPERJ/Brazil (to Valdirene S. Muniz, Renata Baptista-dos-Reis, and Josiane S. Neves) supported this work.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 980983, 10 pages
http://dx.doi.org/10.1155/2015/980983

Research Article

Exposure to Allergen Causes Changes in NTS Neural Activities after Intratracheal Capsaicin Application, in Endocannabinoid Levels and in the Glia Morphology of NTS

Giuseppe Spaziano,[1] Livio Luongo,[1] Francesca Guida,[1] Stefania Petrosino,[2] Maria Matteis,[1] Enza Palazzo,[3] Nikol Sullo,[1] Vito de Novellis,[1] Vincenzo Di Marzo,[2] Francesco Rossi,[1] Sabatino Maione,[1,2] and Bruno D'Agostino[1]

[1] *Endocannabinoid Research Group, Section of Pharmacology, "L. Donatelli," Department of Experimental Medicine, School of Medicine, Second University of Naples, Via Costantinopoli 16, 80138 Naples, Italy*
[2] *Endocannabinoid Research Group, Institute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche, Pozzuoli, 80078 Naples, Italy*
[3] *Department of Anaesthesiology, Surgery and Emergency, Second University of Naples, Piazza Luigi Miraglia 2, 80138 Naples, Italy*

Correspondence should be addressed to Sabatino Maione; sabatino.maione@unina2.it
and Bruno D'Agostino; bruno.dagostino@unina2.it

Received 16 June 2014; Revised 25 September 2014; Accepted 6 October 2014

Academic Editor: Troy Carlo

Allergen exposure may induce changes in the brainstem secondary neurons, with neural sensitization of the nucleus solitary tract (NTS), which in turn can be considered one of the causes of the airway hyperresponsiveness, a characteristic feature of asthma. We evaluated neurofunctional, morphological, and biochemical changes in the NTS of naive or sensitized rats. To evaluate the cell firing activity of NTS, in vivo electrophysiological experiments were performed before and after capsaicin challenge in sensitized or naive rats. Immunohistochemical studies, endocannabinoid, and palmitoylethanolamide quantification in the NTS were also performed. This study provides evidence that allergen sensitization in the NTS induced: (1) increase in the neural firing response to intratracheal capsaicin application, (2) increase of endocannabinoid anandamide and palmitoylethanolamide, a reduction of 2-arachidonoylglycerol levels in the NTS, (3) glial cell activation, and (4) prevention by a Group III metabotropic glutamate receptor activation of neural firing response to intratracheal application of capsaicin in both naïve and sensitized rats. Therefore, normalization of ovalbumin-induced NTS neural sensitization could open up the prospect of new treatments based on the recovery of specific brain nuclei function and for extensive studies on acute or long-term efficacy of selective mGlu ligand, in models of bronchial hyperreactivity.

1. Introduction

Bronchial hyperresponsiveness (BHR), a characteristic feature of asthma, may be exacerbated by various local inflammatory mediators released by repeated exposures to allergen [1, 2]. Over the last few years, it has been shown that several inflammation-generated mediators induce long-term functional modifications of the sensory airway neural pathways in rodent and primate models of asthma: neuroplastic changes in the peripheral airway afferent nerves as well as in the brainstem secondary neurons and/or motor vagus output neurons have been demonstrated [3]. The direct consequence of neuroplasticity in the brainstem nucleus of solitary tract (NTS) or the dorsal motor nucleus of vagus is mainly represented by neural sensitization which in turn may be considered one of the causes of the BHR to various bronchoconstrictor stimuli [4].

Over the last decade, evidence has accumulated on the complex biomolecular mechanisms related to neural sensitization and plasticity, which are critical for a variety of phenotypic changes in neuron activities [5]. These functional

changes are considered to be at the basis both of several physiological events such as memory and learning [5, 6] and of many pathological conditions, such as chronic pain syndromes [7]. Indeed, enduring neuropathic or inflammatory pain is a well-characterized pathophysiological condition in which a direct parallel between persistent exposure to excitatory/inflammatory neurotransmitters and the increased excitability of spinal post-synaptic neurons has been clearly shown [8–11]. Many studies have proposed an analogy between airway hyperresponsiveness and hyperalgesia. Considering that the endovanilloid oleoylethanolamide excites sensory vagal neurons via TRPV1 receptors [12] and that BHR mediated by several stimuli [13, 14] is abolished following chronic treatment with capsaicin; sensory nerves can represent a common pathway by which many stimuli can induce BHR. These studies are consistent with the hypothesis that "sensitization" of airway sensory nerves may contribute toward this phenomenon [15].

Further confirmation of a similarity between the neural adaptive mechanisms for airway neural sensitization and the establishment chronic pain is the fact that both phenomena share the same neurotransmitters and neuromodulators (i.e., glutamate, SP, GABA, endocannabinoids, etc.) at both peripheral (lung and trachea) and brainstem levels [3, 16, 17]. In particular, the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) and their main cannabinoid CB1 and CB2 receptors have been identified in the NTS [18], where another molecular target of anandamide, the transient receptor potential vanilloid type-1 (TRPV1) channel, is also abundantly coexpressed with CB1 receptors [17].

Moreover, the anandamide congener palmitoylethanolamide (PEA), which activates peroxisome proliferator-activated receptor (PPAR)-α, can also enhance anandamide actions at CB1 and TRPV1 receptors [19]. Importantly, NTS TRPV1 channel stimulation by capsaicin was shown to induce the cough reflex in the guinea pig [20], whereas CB1 receptors in this nucleus seem to be more involved in the control of emesis, oesophageal sphincter relaxation, and baroreflex-evoked sympathoinhibition [17, 18, 21, 22].

Based on these considerations, we have evaluated some functional, morphological, and biochemical changes occurring in the NTS following airway sensory nerve activation in naive and ovalbumin-sensitized rats. In particular, we evaluated (i) the responsiveness of the intrinsic NTS neurons by intratracheal application of capsaicin; (ii) the levels of the two major endocannabinoids, anandamide, and 2-AG and of the cannabinoid receptor-inactive PEA; (iii) the morphological changes in NTS microglia and astroglia. Group III metabotropic glutamate receptors include mGlu4, mGlu6, mGlu7, and mGlu8 mainly located on presynaptic terminals where they modulate neurotransmitter release. L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), L-serine-O-phosphate (L-SOP), and (1S, 2R)-1-amino-phosphonomethylcyclopropane carboxylic acid (1S, 2R)-APCP are broad spectrum agonists whereas L-AP4 and L-SOP α-methyl analogs, (S)-α-methyl-2-amino-4-phosphonobutanoic acid (MAP4), and (RS)-α-methylserine-O-phosphate (MSOP) behave as antagonists. Since group III mGlu receptor modulate local neurokinins and glutamate releases [23], we also analyzed their roles in NTS neuron activities before and after capsaicin-induced C-fibers afferent nerve activation.

2. Materials and Methods

2.1. Animals. Male Norway brown rats (250–300 g) were housed 3 per cage under controlled illumination (12 : 12 h light : dark cycle; light on 06.00 h) and environmental conditions (ambient temperature 20–22°C, humidity 55–60%) for at least 1 week before the commencement of experiments. Rat chow and tap water were available *ad libitum*. The experimental procedures were approved by the Animal Ethics Committee of the Second University of Naples. Animal care was in compliance with Italian (D.L. 116/92) and EEC (O.J. of E.C. L358/1 18/12/86) regulations on the protection of laboratory animals. All efforts were made to minimise animal suffering and to reduce the number of animals used.

2.2. Sensitization. The rats were sensitized by a subcutaneous (sc) injection of 0,66 mL of a suspension of 1 mg OVA plus 300 mg of aluminium hydroxide in 0,9% NaCl solution (saline) [24]. Naïve rats received saline only. This was considered Day 1 of sensitization. Seven days after sensitization, the animals were boosted subcutaneously (sc) with an identical injection of ova suspension. Twenty one days after the initial injection**,** animals were challenged with 5% aerosolized OVA. OVA was aerosolized for 5 min using an ultrasonic nebuliser and nebuliser control unit (Buxco Electronics). On the day 22, 24 hours after the OVA challenge, bronchopulmonary function was performed. Control animals were challenged with 0.9% saline solution. To evaluate the successful of OVA sensitization, five rats of each group (sensitized and naive) were used to assess airway responsiveness. Animal were anaesthetized by an i.p. injection of urethane (1.3 g/kg, i.p.) and lung function was assessed 30 min later. The anaesthetized rats were exposed to sterile saline for 2 min and lung functions were recorded. Airway responsiveness (R_L) was measured following aerosol administration of double concentrations of metacholine for 30 s and measurements of respiratory parameters were taken every minute for 5 min. Peak value of R_L was measured after each concentration and the challenge was stopped at 128 mg/mL metacholine. We have measured the concentrations of metacholine inducing 200% increase of R_L over the initial baseline ($EC_{200}R_L$).

2.3. Experimental Protocol. Groups of 5 animals per treatment were used with each animal being used for one treatment only.

A group of naive rats was implanted with guide cannulae and received an intracerebral microinjection of 2 microliters of ACSF and served as a control of the intracerebral drug microinjection.

For the in vivo extracellular recording, naive and sensitized rats were grouped as follows.

(a) Groups of naive or sensitized rats received intracerebral administration of L-AP4 (2 and 4 nmol/rat) alone or L-AP4 (4 nmol/rat) in combination with MSOP (100 nmol/rat). When L-AP4 was administered in

combination with MSOP, the latter was centrally delivered 5 min before the administration of L-AP4.

(b) Groups of naive or sensitized rats received intracerebral administration of MSOP (100 and 300 nmol).

All groups received intratracheal capsaicin challenge (300 pg in 20 μL) or respective vehicle. In a separate set of experiments, groups of sensitized and naive rats were killed with a lethal dose of pentobarbital and decapitated for assay of endocannabinoid content and for the immunohistochemistry analysis in the NTS area. The doses were chosen according to previous data [25].

2.4. Preliminary Surgical Preparations. Each rat was anaesthetized with an i.p. injection of pentobarbital (50 mg/kg). A catheter was introduced into the jugular vein for administering saline or for the continuous infusion of propofol (5–10 mg/kg/h) to maintain a constant anaesthesia. Trachea was cannulated below the larynx, and a tiny catheter was also connected to a side-part of that cannula to allow intratracheal vehicle or capsaicin (300 pg in 20 μL) application. The cervical vagus nerve ipsilateral to the recording site was isolated (mainly the right side) for the placement of the stimulating electrode. In order to perform administrations of drug or respective vehicle (artificial cerebrospinal fluid, ACSF, composition in mM: KCl 2.5; NaCl 125; $MgCl_2$ 1.18; $CaCl_2$ 1.26) into the cerebral lateral ventricle, a 23-gauge, 12 mm-long stainless steel guide cannula was stereotaxically lowered until its tip was 1.5 mm above the ventricle by applying coordinates from the atlas of Paxinos and Watson [26] (A: 0.92 mm and L: 1.5 mm from bregma, V: 2.9 mm below the dura).

These coordinates were chosen in order to have enough space to allow stereotaxic manipulation for the positioning of both the guide cannula for drug microinjection and of the tungsten electrode for the in vivo NTS cell recording. The guide cannula was anchored with dental cement to a stainless steel screw in the skull. We used a David Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) with the animal positioned on a homeothermic temperature control blanket (Harvard Apparatus Limited, Edenbridge, Kent). The guide cannula for drug microinjection was implanted on the same day as the electrophysiological recording. Direct intracerebral administration of drugs or respective vehicle was conducted with a stainless steel cannula connected by a polyethylene tube to a SGE 1-microlitre 26-gauge syringe, inserted through the guide cannula and extended 1.5 mm beyond the tip of the guide cannula to reach the cerebral ventricle. Volumes of 2 μL drug solutions or vehicle were injected into the ventricle over a period of 60 s and the injection cannula gently removed 2 min later.

2.5. NTS Extracellular Recording. After implantation of the guide cannula into the cerebral ventricle, a tungsten microelectrode was stereotaxically [26] lowered through a small craniotomy to record the activity of the airway-related NTS neurons before and after intratracheal application of capsaicin. These neurons were identified by stimulating the vagus nerve (200–600 μA, 0.5–0.8 ms pulses) at 1 Hz during the slow (1 μm s) electrode lowering within the NTS [14, 27]. Extracellular single-unit recordings were made in the NTS with glass insulated tungsten filament electrodes (3–5 MΩ) (FHC Frederick Haer & Co., ME, USA) using the following stereotaxic coordinates: 3–3.6 mm caudal to lambda, 1–1.5 mm lateral, and 7.7–8.1 mm depth from the surface of the brain [26]. The recorded signals were amplified and displayed on analog and digital storage oscilloscope to ensure that the unit under study was unambiguously discriminated throughout the experiment. Signals were also fed into a window discriminator, whose output was processed by an interface (CED 1401) (Cambridge Electronic Design Ltd., UK) connected to a Pentium III PC. Spike2 software (CED, version 4) was used to create peristimulus rate histograms online and to store and analyse digital records of single-unit activity offline. Configuration, shape, and height of the recorded action potentials were monitored and recorded continuously, using a window discriminator and Spike2 software for on-line and off-line analysis. Once an NTS unit was identified from its background and tracheal/vagus stimulation activity, we optimised spike size before all treatments. This study only included neurons whose spike configuration remained constant and could clearly be discriminated from activity in the background throughout the experiment, indicating that the activity from one neuron only and from that same neuron was measured. Only one neuron was recorded in each rat and the recording RVM site was marked with a 20 μA DC current for 20 s.

2.6. Endocannabinoid Extraction and Quantification

2.6.1. Analysis of Endocannabinoid Contents. Anaesthetized rats were decapitated and their brains were rapidly removed and immersed in oxygenated ice-cold artificial cerebrospinal fluid. A block of tissue containing the NTS was cut using a vibrotome (Vibratome 1500, Warner Instruments, CT, USA). A brainstem slice of 2–2.5 mm was cut throughout the medulla containing the NTS region and using the following stereotaxic coordinates: 3–3.6 mm caudal and 1–1.5 mm lateral to lambda [26]; the right and left NTS from the same rat were isolated under microscope (M650, Wild Heerbrugg, Switzerland) and pooled. Tissues were homogenized in 5 vol of chloroform/methanol/Tris HCl 50 mM (2 : 1 : 1) containing 50 pmol of d_8-anandamide, d_4-palmitoylethanolamide, and d_5-2-AG. Deuterated standards were synthesized from d_8 arachidonic acid and ethanolamine or arachidonic acid and d_5-glycerol or d_4-palmitic acid and ethanolamine. Homogenates were centrifuged at 13,000 g for 16 min (4°C), the acqueous phase plus debris were collected and extracted again twice with 1 vol of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized samples were then stored frozen at −80°C under nitrogen atmosphere until analyzed and were resuspended in chloroform/methanol 99 : 1 by vol. The solutions were then purified by open bed chromatography on silica as described by Maione et al. [28]. Fractions eluted with chloroform/methanol 9 : 1 by vol. (containing anandamide, pamitoylethanolamide, and 2 AG) were collected and the excess solvent evaporated with a rotating

evaporator, and aliquots analyzed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionisation/mass spectrometry (LC APCI-MS) carried out under conditions described previously [28] and allowing the separations of 2-AG, palmitoylethanolamide, and anandamide. MS detection was carried out in the selected ion monitoring mode using m/z values of 356 and 348 (molecular ion+1 for deuterated and undeuterated anandamide), 304.0 and 300.0 (molecular ion+1 for deuterated and undeuterated palmitoylethanolamide), and 384.35 and 379.35 (molecular ion+1 for deuterated and undeuterated 2 AG). The area ratios between signals of deuterated and undeuterated anandamide varied linearly with varying amounts of undeuterated compounds. Anandamide, palmitoylethanolamide, and 2 AG levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas.

2.7. Immunohistochemistry. Under pentobarbital anaesthesia animals were transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was excised, postfixed for 4 hr in the perfusion fixative, cryoprotected for 72 h in 20% sucrose in 0.1 M phosphate buffer, and frozen in O.C.T embedding compound. 20 μm transverse sections were cryostat cut and thaw-mounted onto glass slides. The NTS was identified based on the Paxinos and Watson Atlas coordinates (1986) [26]. Slides were incubated overnight with primary antibody solutions for the microglial cell marker Iba-1 (Rabbit anti-ionized calcium binding adapter molecule 1; 1 : 1000; Wako Chemicals, Germany), the astrocytes marker GFAP (Glial fibrillary acidic protein; 1 : 1000; DAKO, USA). Following incubation sections were washed and incubated for 3 hr with secondary antibody solution (goat anti-rabbit, IgG-conjugated Alexa Fluor 488; 1 : 1000; Molecular Probes, USA). Slides were washed, cover-slipped with Vectashield mounting medium (Vector Laboratories, USA), and visualised under a Zeiss Axioplan 2 fluorescent microscope.

Quantitative assessment was carried out by determining the intensity of positive profiles for each marker within a fixed area of the NTS. A box measuring 10^4 μm^2 was placed onto areas of the lateral, central, and medial NTS and the intensity of positive profiles within this area recorded by a AxioVision Rel. 4.6 program. This measurement protocol was carried out on three NTS sections from each animal.

2.8. Drugs. Capsaicin (Sigma-Aldrich, Milano Italy) was dissolved in a solution consisting of ethanol and water (6 : 4).

L-2-Amino-4-phosphonobutyric acid and (RS)-α-methylserineo-phosphate were purchased by Tocris Bioscience, Bristol, UK, and dissolved in ACSF.

2.9. Statistics. For electrophysiological study, the single extracellular recording (action potentials) was analysed offline from peristimulus rate histograms using Spike2 software (CED, version 4). The neuron responses, before and after capsaicin-induce stimulation or following intracerebroventrolateral vehicle or drug microinjections, were measured and expressed as spikes/sec (Hz). In particular, basal values were obtained by averaging the activities recorded 10 min before drug applications. Data are presented as mean ± standard error (S.E.) of changes in neuron responses (extracellular recordings).

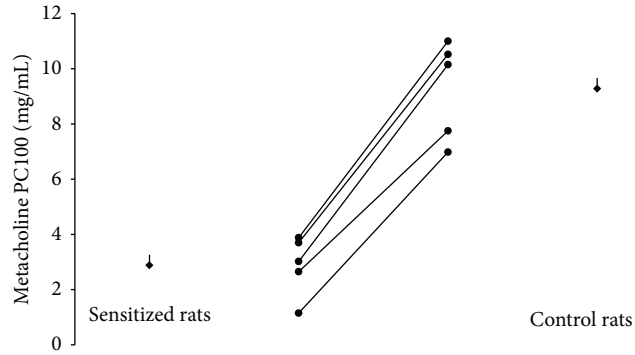

Figure 1: Airway responsiveness to methacholine in ovalbumin (sensitized) or saline treated rats (control) ($n = 5$). $^{**}P < 0.01$ compared with control group. Circles represent single values and squares represent mean values.

Statistical comparisons of values from different treated groups of rats were made using the two-way analysis of variance (ANOVA) for repeated measures followed by the Tukey/Kramer test for post hoc comparisons. Comparisons between pre- and posttreatment ongoing activity and capsaicin-related cell burst were performed by applying the nonparametric Wilcoxon matched-pairs signed rank test.

Mean values for each group were then compared using Student's t-test. $P < 0.05$ was set as the level of statistical significance.

The amounts of endocannabinoids were expressed as picomoles or nanomoles per gram of wet tissue extracted and were compared by ANOVA followed by Bonferroni's test.

3. Results

3.1. Airway Responsiveness and Endocannabinoid Levels Measurements of Naive and Sensitized Rats. Baseline absolute value of R_L and C_{dyn} was not significantly different between two groups.

In sensitized rats, OVA aerosol caused an acute bronchoconstriction, with an approximately threefold greater increase in R_L and decrease in C_{dyn} respect to an aerosol of saline solution (data not shown). OVA challenge exposure resulted in an increase of airway responsiveness to inhaled histamine, approximately fourfold ($P < 0.01$) when compared with saline challenge (PC_{100}: 69 mg/mL; 18 mg/mL saline and ova groups, resp.) (Figure 1). Moreover, sensitization caused an enhancement of the endogenous TRPV1/CB1 "hydrid" agonist, anandamide, and of the endogenous PPARα agonist palmitoylethanolamide levels in the NTS area, whilst lowering the levels of the CB1-selective endocannabinoid 2-AG (Table 1).

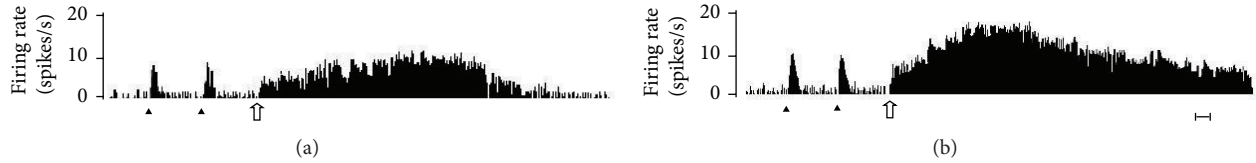

Figure 2: Example of ratemeter records which illustrate the spontaneous activity of NTS neurons before and after capsaicin administration (hollow arrow). (a) control rat, (b) ovalbumin-sensitized rat. A single oscilloscope trace (2 min recording) shows spontaneous activity of a single unit (long black arrow) immediately before and after intratracheal capsaicin application. Small black arrowheads indicate single vagal stimulations. Scale bar = 1 min.

Table 1: Endocannabinoid levels in the NTS area.

	NTS	
	Control	Sensitized
Anandamide (pmol/g)	32.0 ± 3.0	49.9 ± 3.1*
2-AG (nmol/g)	7.4 ± 0.1	6.5 ± 0.1*
Palmitoylethanolamide (pmol/g)	961.4 ± 16.4	1187.8 ± 43.4*

Data are means ± SEM of $n = 4$ separate experiments. * $P < 0.05$ as assessed by ANOVA followed by Bonferroni's test.

3.2. The Effect of Intratracheal Capsaicin on Airway-Related NTS Neuron Activities in Naive or Sensitized Rats. The results are based on airway-related NTS neurons (group size = 5; one cell recorded from each animal per treatment) at a depth of 7.7–8.1 μm from the surface of the brain, the estimated location of the neurons being within the NTS. All recorded neurons showed very little spontaneous activity (they frequently paused completely during 20–50 s) and discharged with a mean frequency of 0.7 ± 0.03 spikes/s. These neurons were identified by an increased burst of activity just after vagus nerve stimulation (Figure 2).

Intratracheal application of capsaicin (300 pg in in 20 μL) induced an increase in the firing activity of the airway-related NTS neurons in naive rats, which was maximal (9.9 ± 0.7 spikes/s) 20 min after the administration of capsaicin (Figure 2(a)). In sensitized rats, intratracheal application of capsaicin induced a higher increase in the firing activities (12.4 ± 0.6 spikes/s) of the airway-related NTS neurons than it did in naive rats. Unlike the naive rats, we did not observe any recovery in the sensitized rats during the observation period (60 min post-capsaicin) (Figure 2(b)).

3.3. The Effect of L-AP4 on Capsaicin-Induced Change on the Airway-Related NTS Neuron Activities in Naive and Sensitized Rats. Intracerebroventricular microinjections of L-AP4 (2–4 nmol/rat) did not induce any effect on the basal value of airway-related NTS cells ongoing activities (data not shown).

The highest doses of L-AP4 (4 nmol/rat) prevented the capsaicin-induced increase in the airway-related NTS neuron activities in both naive and sensitized rats (Figures 3(a) and 3(b)). The effects of L-AP4 (4 nmol/rat) were prevented by pretreatment with MSOP (100 nmol/rat), which *per se* did not significantly change the airway-related NTS neuron (Figures 3(a) and 3(b)).

3.4. Immunohistochemistry. Immunoreactivity (IR) for the microglial cell marker Iba-1 was observed in the NTS of control and sensitized animals. In sensitized animals, the increased expression of Iba-1 and specific morphological changes, such as the increased thickness of cell bodies and process retraction, suggest activation of microglia. In particular, quantitative analysis of Iba-1 IR revealed a significant increase in the intensity of Iba-1 positive cells in the NTS of sensitized rats (79.9 ± 4.6 arbitrary units), in comparison to naive animals (58.9 ± 3.8 arbitrary units). As far as the analysis of astrocyte activity is concerned, IR for marker GFAP was evaluated in the NTS of control and sensitized animals. In sensitized animals, we observed an increased expression of GFAP and specific morphological changes, such as increased astrocyte cell body and process thickness, assuming reactive astrogliosis following sensitization to ovalbumin. In particular, GFAP IR quantitative analysis revealed a significant increase in the intensity of GFAP positive cells in the NTS of sensitized rats (147.1 ± 3.8 arbitrary units), in comparison to control animals (101.6 ± 3.3 arbitrary units) (Figure 4).

4. Discussion

This study shows that ovalbumin-induced sensitization increases: (1) the NTS neural firing response to intratracheal capsaicin application, (2) the endocannabinoid anandamide level, and (3) astro- and microgliosis in the NTS. Moreover, we also show that the intracerebroventricular application of a Group III metabotropic glutamate receptor agonist prevents the neural firing response to the intratracheal application of capsaicin in both naïve and sensitized rats. The overall hypothesis linking these different findings to the generation of bronchial hyperresponsiveness (BHR) is based on the possibility that peripheral nerve sensitization such as, for example, during persistent inflammation, may induce long-lasting pathophysiological modifications in the NTS neural and glial cell functioning. Indeed, in a similar way to the changes observed in the spinal cord in chronic pain [7, 9], we suggest that, also in this case, a higher discharge of the afferent sensitized neurons may increase the release of excitatory neurotransmitters (i.e., glutamate and CGRP) in the NTS responsible for neurons, astrocytes, and microglia phenotypic modifications [29]. Importantly, pathophysiological conditions like chronic pain or inflammation are associated with alterations in the levels of some on-demand produced endocannabinoid/endovanilloids such as anandamide, PEA, or 12-lipoxygenase products (i.e., 12-HPETE) [30–33].

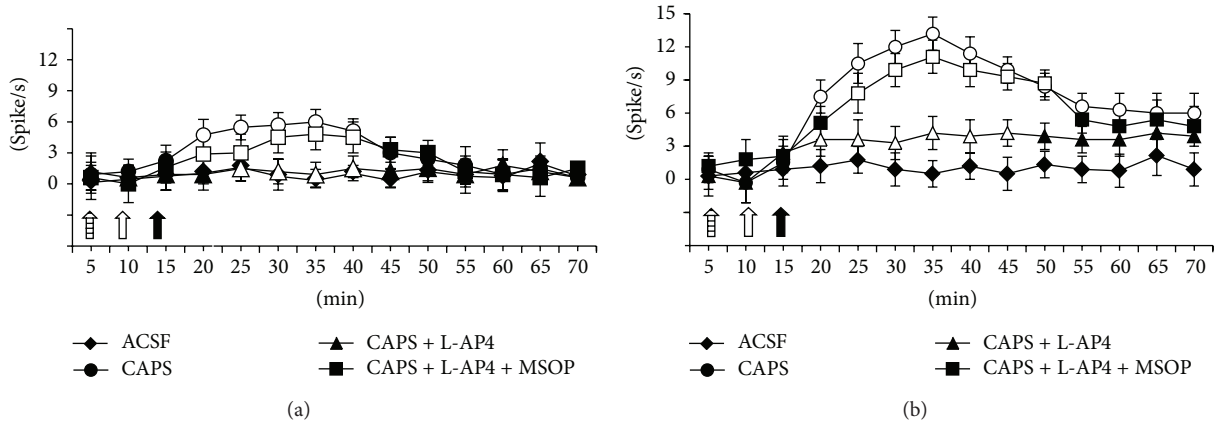

Figure 3: Effect of vehicle (20% DMSO in ACSF) or capsaicin (300 pg in 20 μL) in naïve (a) and sensitized (b) rats. L-AP4 (4 nmol/rat) (hollow arrow) prevented the capsaicin (full arrow) induced increase in the airway-related NTS neuron ongoing activities. This effect of L-AP4 (4 nmol/rat) was prevented by pretreatment with MSOP (100 nmol/rat) (arrow with lines). Each point represents the mean ± SEM of five rats per group. Values statistically (P value < 0.05) significant versus the respective control were indicated as open symbols.

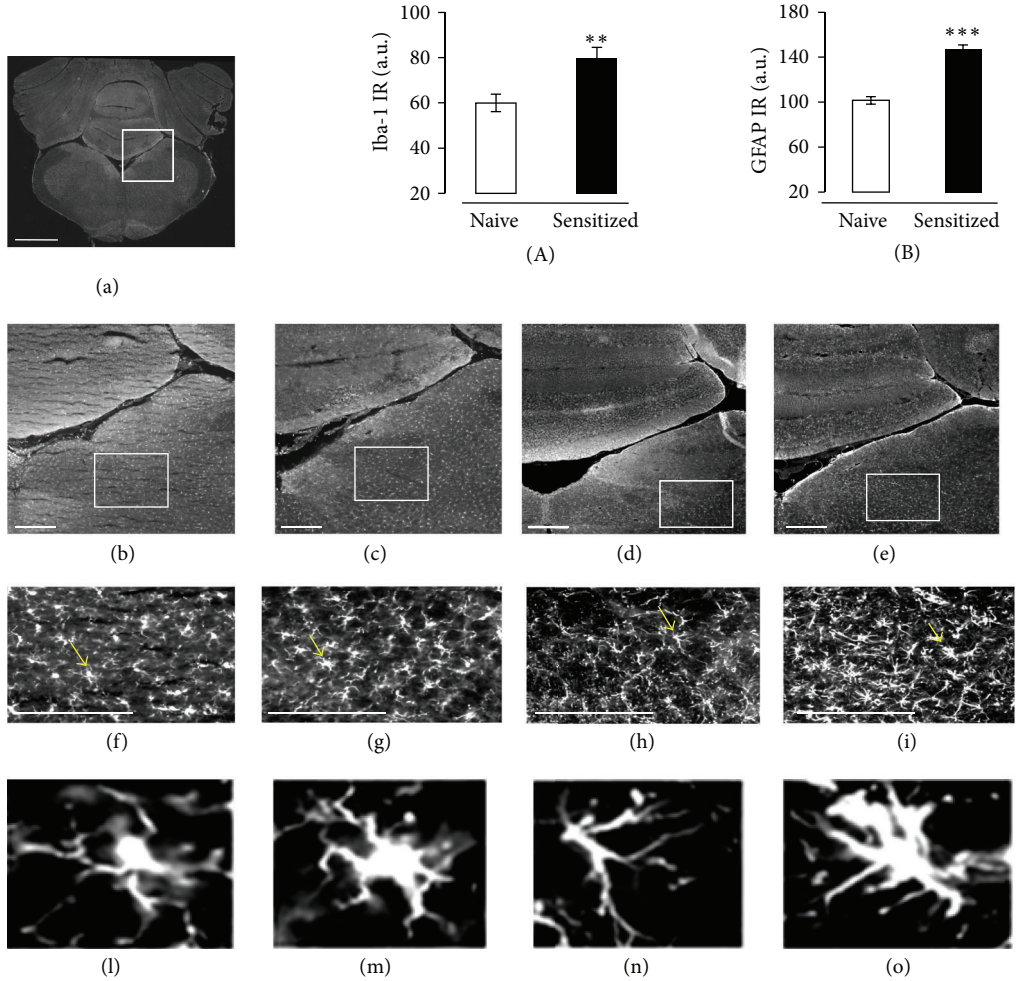

Figure 4: (a) Low magnification of NTS brain area and schematic representation (see Paxinos and Watson, 1986 [26]) of the area beside. (b, f, l) Iba-1 IR in NTS of a naive rat. (c, g, m) Iba-1 IR in NTS of sensitized rat to ovalbumin. (d, h, n) GFAP IR in NTS of a naive rat. (e, i, o) GFAP IR in NTS of sensitized rat. High magnification of Iba-1 + profiles (l, m) and GFAP + profiles (n, o) arrows. Scale bars = 100 μm. (A, B) Quantitative analysis of Iba1 and GFAP staining in NTS reveals significantly increased numbers of Iba-1 and GFAP-positive cells in the NTS after ovalbumin sensitization. Data represented as mean ± SEM, $n = 3$ rats per group. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group, one-way ANOVA, post hoc Tukey.

Consistently with this possibility, the increase in the NTS neural firing response to intratracheal application of capsaicin in sensitized animals has led us to believe that these cells might be hyperactive airway-activated NTS neurons [34]. Considering that in this study intratracheal capsaicin induced a higher and long-lasting firing discharge of the airway-related NTS neurons, one might speculate that similarly to previous findings [35–37], chronic allergen challenge can lead to persistent inflammation and activation of afferent vagal fibers modulating the activity of the airway-activated NTS neurons. Indeed, persistent stimulation on the small-diameter nerve fibers (i.e., C-fibers and Aδ-fibers) by several direct and indirect acting chemical mediators in the lung may be responsible for afferent neuron sensitization and for phenotypic modifications in NTS cell functioning [29]. In line with this study, NTS neural sensitization in slices of asthmatic primates was shown by Chen and colleagues [38], and it may be possible that the electrophysiological effects observed here could also be related to the increased activity of microglia and astrocytes in the NTS that, in turn, can alter the synaptic plasticity. Accordingly, with the idea that glia plays critical role in determining or sensing neuronal well-being and is capable of shaping neural activities either in healthy or in several pathological brain conditions [39–41], our current findings demonstrate the occurrence of gliosis in the NTS of albumin-sensitized rats. However, it is also intriguing that in many cases gliosis has two faces, protective or deleterious, and understanding of the rules governing this duality is still in its initial stages [42]. Nevertheless, there is evidence to suggest that neurons and glia mutually affect their functioning through complex, not fully explored mechanisms [39, 43] generating alterations in the levels of excitatory (i.e., glutamate and CGRP) and inhibitory (i.e., GABA and endocannabinoids) neurotransmitters. In particular, the recently identified endovanilloids/endocannabinoids are capable of glia activation/differentiation and play roles in neurodegenerative disorders accompanied by microglial activation [44–46]. Regarding endocannabinoid involvement in the modulation of the NTS neural activities, it has been shown that, by acting on presynaptic cannabinoid CB1 receptors, they inhibit both excitatory and inhibitory signalling in the NTS [47]. In contrast, by activating TRPV1 receptors, the endocannabinoid anandamide stimulates glutamatergic signalling [48], with subsequent (1) stimulation of GABA release in this nucleus and a subsequent decrease in NTS neuronal firing [48]; and/or (2) stimulation of output neurons; two effects that would reduce and increase bronchoconstriction (in the latter case via reflex output disinhibition and increased bronchoconstrictive reflexes), respectively. However, the effect of CB1 receptor activation has so far mostly been related to the control of emesis, lower visceral functions, and blood pressure [17, 18, 21, 22].

In order to preliminarily evaluate a role of the endocannabinoid system at NTS level, we measured the content of the two more representative endocannabinoids, anandamide, and 2-AG, as well as of the PPAR-α endogenous agonist that also enhances anandamide actions at CB1 and TRPV1 receptors, palmitoylethanolamide (PEA). Here, we show that airway sensitization is accompanied by a statistically significant enhancement of anandamide and PEA, whereas it induced a reduction in 2-AG levels in the NTS. Although endocannabinoids like anandamide might inhibit synaptic transmission via CB1 receptors in the NTS [47], we found that the overall endocannabinoid tone in this nucleus might remain unchanged or have even decreased following airway sensitisation, because of the opposite changes of anandamide and 2-AG levels and the fact that basal 2-AG levels are higher than AEA levels. Indeed, a reduction rather than an increase in cannabinoid receptor activity would be more in line with the increased microglial and glial cell density observed here in the NTS following ovalbumin sensitisation, since (1) CB1 receptor activation was recently shown to inhibit gliosis induced by a β-amyloid peptide [49]; (2) both CB1 and CB2 receptor activations were found to inhibit the release of proliferation- and motility-inducing cytokines from astrocytes [50]; and (3) CB2 receptor agonists inhibit microglial cell activation in animal models of neuroinflammatory disorders [51, 52]. On the other hand, the elevated levels of anandamide and PEA in the NTS might underlie the increased microglial density observed in this nucleus following ovalbumin sensitisation, since these two neurotransmitters synergistically stimulate microglial migration via non-CB1 non-CB2 receptors [53]. Conversely, since microglial cells produce more PEA and anandamide than 2-AG [54], the observed changes in NTS levels of these compounds might be due to the elevated active microglia found in sensitised rats. It is worth noting that opposing regulation on anandamide and 2-AG levels is not unprecedented in literature, and a recent finding indicates that, by activating TRPV1, anandamide might in fact reduce 2-AG biosynthesis in the striatum [55, 56].

The above observations suggest that anandamide may preferentially activate TRPV1 in the NTS than cannabinoid receptors, as has been observed in the periaqueductal grey following administration of intermediate doses of an inhibitor of anandamide enzymatic hydrolysis [28]. In the presence of (1) concomitantly elevated levels of PEA, which potentiates anandamide effects at TRPV1 [57], and is unlikely to act via PPAR-α (which has never been described as being expressed in the NTS), and (2) reduced CB1 tone, which disinhibits TRPV1 activity [56], anandamide activity at these channels might be enhanced further. The subsequent enhancement of glutamatergic signalling would either stimulate NTS output neuron activity, thus contributing to sensitisation-induced airway hyperresponsiveness. Alternatively, it could enhance GABAergic signalling and hence counteract NTS neuronal firing [48] and bronchoconstriction, representing an adaptive response to sensitization. The former possibility is supported by our finding that the blockade of glutamatergic signalling by Group III metabotropic glutamate receptors activation reduces capsaicin-induced elevation of NTS neuronal activity. However, since prolonged activation of TRPV1 can also cause its desensitisation, it is possible that ovalbumin-induced elevation of anandamide, by desensitising TRPV1, acts in a similar way to Group III mGlu receptor activation.

Indeed, vagal brainstem circuits seem to be organized in such a way that Group II subtype receptors (mGlu2

and mGlu3) are expressed on GABAergic and glutamatergic intrinsic NTS neurons, whereas Group III mGlu receptors seem to be mainly expressed on glutamatergic nerve terminals impinging on output preganglionic neurons [23]. While NMDA and AMPA/Kainate receptor contribute to the excitatory inputs and in the activity-dependent plastic changes of NTS during airway hyperreactivity [58, 59]. Group III mGlu receptors have been shown to significantly contribute to the depression of autonomic signal transmission by attenuating the presynaptic release of glutamate and neurokinins [38]. In this study, the intracerebroventricular administration of L-AP4, the relative selective Group III metabotropic glutamate receptor agonist, prevented the airway-related NTS neuron discharges induced by intratracheal capsaicin in naïve and sensitized rats and confirming their modulatory role in glutamate release. Moreover, it is worth noting that the same dose of L-AP4 prevented capsaicin-induced NTS cell discharges in both naïve and sensitized rats. This observation allows us to speculate that the occurrence of desensitization mechanisms for Group III mGlu receptors might be excluded in the sensitization model applied here. If this may represent an additional advantage in the potential management of bronchial hyperreactivity with selective mGlu receptor ligands, it is obvious that extensive studies are needed in order to examine their possible systemic use and efficacy in different in vivo models of bronchial hyperresponsiveness or asthma in more detail.

5. Conclusions

In conclusion, we found that the allergen sensitization in the NTS induced (1) an increase in the neural firing response to intratracheal capsaicin application, (2) an endocannabinoid anandamide increase, and (3) glial cell activation. Although the pathophysiological significance of these different findings remains to be assessed, they could however be relevant to the altered NTS neurotransmitter and cellular morphofunctional changes, which in turn might be collectively involved in the long-lasting NTS cell phenotypic modifications. The overall hypothesis is that the different findings are not independent events, but are direct consequence of the peripheral nerve sensitization which is in turn capable of inducing long-lasting airway-related NTS neural sensitization and hence bronchial hyperresponsiveness. Interestingly, it was also found that acute intracerebroventricular application of a Group III metabotropic glutamate receptor agonist prevented a neural firing response to intratracheal application of capsaicin in both naïve and sensitized rats. Normalization of ovalbumin-induced NTS neural sensitization opens up the prospect of new treatments based on the recovery of specific brain nuclei function and for extensive studies to examine the acute or long-term efficacy of selective mGlu ligand in specific models of bronchial hyperreactivity in greater detail.

Abbreviations

NTS: Nucleus solitary tract
OVA: Ovalbumin
BHR: Bronchial hyperresponsiveness
R_L: Lung resistance
C_{dyn}: Dynamic compliance
TRPV1: Transient receptor potential vanilloid type 1.

Conflict of Interests

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

Authors' Contribution

Giuseppe Spaziano and Livio Luongo contributed equally to the work presented in this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 264270, 12 pages
http://dx.doi.org/10.1155/2014/264270

Research Article

Protective Role of 5-Lipoxigenase during *Leishmania infantum* Infection Is Associated with Th17 Subset

Laís Amorim Sacramento,[1] Fernando Q. Cunha,[1,2] Roque Pacheco de Almeida,[2] João Santana da Silva,[1] and Vanessa Carregaro[1]

[1] *Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, Avenida Bandeirantes 3900, 14049-900 Ribeirão Preto, SP, Brazil*
[2] *Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil*

Correspondence should be addressed to Vanessa Carregaro; vcarregaro@usp.br

Received 20 June 2014; Revised 2 September 2014; Accepted 2 September 2014; Published 21 September 2014

Academic Editor: Ruxana Sadikot

Visceral leishmaniasis (VL) is a chronic and fatal disease caused by *Leishmania infantum* in Brazil. Leukocyte recruitment to infected tissue is a crucial event for the control of infections such as VL. Leucotriens are lipid mediators synthesized by 5-lipoxygenase (5-LO) and they display a protective role against protozoan parasites by inducing several functions in leucocytes. We determined the role of 5-LO activity in parasite control, focusing on the inflammatory immune response against *Leishmania infantum* infection. LTB_4 is released during *in vitro* infection. The genetic ablation of 5-LO promoted susceptibility in highly resistant mice strains, harboring more parasites into target organs. The susceptibility was related to the failure of neutrophil migration to the infectious foci. Investigating the neutrophil failure, there was a reduction of proinflammatory cytokines involved in the related Th17 axis released into the organs. Genetic ablation of 5-LO reduced the $CD4^+$T cells producing IL-17, without interfering in Th1 subset. *L. infantum* failed to activate DC from 5-$LO^{-/-}$, showing reduced surface costimulatory molecule expression and proinflammatory cytokines involved in Th17 differentiation. BLT_1 blockage with selective antagonist interferes with DC maturation and proinflammatory cytokines release. Thus, 5-LO activation coordinates the inflammatory immune response involved in the control of VL.

1. Introduction

Visceral leishmaniasis (VL) is one of the most severe clinical manifestations of infection with *Leishmania* parasites and it is a major cause of human mortality and morbidity globally; VL is caused by *Leishmania donovani* and *Leishmania infantum* (World Health Organization, 2010).

The host protective response against *Leishmania spp.* is predominantly mediated by cellular immunity mechanisms, which are critical for parasite replication control and disease resolution. Initially, during infection, activated dendritic cells (DCs) modulate inflammatory leucocyte recruitment to the infection foci [1] and the development of the T $CD4^+$ lymphocyte response characterized by robust IFN-γ and IL-17 production [2, 3]. The immune cell recruitment to *Leishmania* infection foci is managed by inflammatory mediators. Chemokines and cytokines have crucial roles in determining the outcome of leishmaniasis [4, 5]. Lipid mediators such as leukotrienes (LTs) are another class of molecules involved in host defense [6].

LTs are generated from the membrane phospholipids of activated innate immune cells, arachidonic acid (AA), through activation of the 5-lipoxygenase (5-LO) enzymes. 5-LO catalyzes oxidation of AA to intermediate 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is enzymatically reduced by 5-LO to the unstable epoxide A_4 leukotriene (LTA_4). LTA_4 could be hydrolyzed to form leukotriene B_4 (LTB_4), which is a potent effector of leukocyte chemotaxis and activation through the BLT_1/BLT_2 G-protein coupled receptors [7]. Inflammatory disease such as asthma [8], allergic rhinitis [9], and rheumatoid arthritis [10] are associated with increased levels of LTB_4.

Studies have demonstrated that LTB_4 is a potent leukotropic, proinflammatory, and immunoregulator mediator [11,

12]. These molecules are involved in the control of infectious diseases, including viral [13, 14], bacterial [15–17], fungal [18–20], and protozoan infections such as those caused by *T. gondii* [21] and *T. cruzi* [22] and nematode infections [23].

Regarding leishmaniasis, LTB_4 displays leishmanicidal activity on macrophages [24] and neutrophils [25] during *in vitro* infection with *L. amazonensis*, through mechanisms dependent on nitric oxide (NO) and reactive oxygen species (ROS), respectively. In addition, the inhibition of the 5-LO pathway promoted high susceptibility to *L. amazonensis* infection, increasing footpad swelling and harbored more parasites in resistant and susceptible infected mice [26]. In *in vitro* macrophage infection with *L. donovani* parasites, the 5-LO enzymatic activity is enhanced, leading to increased amounts of arachidonic acid metabolites [27], and *in vivo, L. donovani* infection promotes an increase of cyclooxygenase and lipoxygenase activities in spleen cells [28]. It was recently reported that *L. infantum in vitro* infection inhibits the LTB_4 signaling pathway dependent on homologous DCSIGN (SIGNR3) during parasite recognition by macrophages [29], suggesting a protective role of LTB_4 during VL induced by *L. infantum*. Their potential in the recruitment of leukocytes that might be involved in parasite restriction is less well understood. We investigated the role of 5-LO activity in the control of experimental VL induced by *L. infantum,* focusing on the inflammatory immune response. We demonstrated that mice lacking 5-LO signaling displayed high susceptibility to *L. infantum* infection because of a commitment on the related Th17 axis released by CD4 T lymphocytes and neutrophil migration to the infection foci.

2. Material and Methods

2.1. Mice. Female wild-type 129/SvEv (WT) mice or mice genetically deficient in 5-LO (129/SvEv-5-$LO^{-/-}$), 18–22 g in weight, were housed in the animal facility of the Department of Biochemistry and Immunology of the School of Medicine of Ribeirão Preto at the University of São Paulo (Brazil) in temperature-controlled rooms (22–25°C); the mice received water and food ad libitum. The experiments were conducted in accordance with the National Institutes of Health (NIH) guidelines on the welfare of experimental animals and with the approval of the Ethics Committee of the School of Medicine of Ribeirão Preto.

2.2. Parasite Culture, Infection, and Parasite Load Estimation. *L. infantum* (isolate HU-UFS14) was cultured in Schneider medium with 20% heat-inactivated fetal bovine serum, 5% penicillin and streptomycin (from Sigma-Aldrich, Saint Louis, MO, USA), and 2% male human urine. The parasite virulence was maintained by serial passages in BALB/c mice. The mice were injected in the retroorbital plexus with 10^7 stationary-phase *L. infantum* promastigotes in 100 μL PBS. The hepatic and splenic parasite burdens were determined using a quantitative limiting dilution assay.

2.3. DC Generation and Infection. Generation of bone marrow-derived dendritic cells (BMDC) was performed as previously described [30]. The BMDCs (1×10^6/mL) cultured in RPMI-1640 supplemented with 10% FBS were infected with *L. infantum* promastigote forms at a 1 : 5 ratio (cells/parasites) for 12, 24, 36, and 48 h. The supernatants were collected to measure LTB_4 by ELISA (BiotrakTm, Amersham Pharmacia Biotech, UK). In some wells, LPS (200 ng/mL) was added to the BMDC culture as the positive control group. The cells were harvested and their surface expression characterized by flow cytometry using antibodies against CD11c, MHC class-II, CD86, and CD40 conjugated to APC, FITC, PECy7, PerCP, and Alexa700, respectively, as well as the control isotypes. The cytokine releases were measured into the supernatant culture using commercial ELISA kits, according to the manufacturer's instructions (BD Biosciences, R&D Systems, Minneapolis, MN, USA). In some experiments, selective BLT_1 leukotriene B_4 receptor antagonist (U-75302, Sigma-Adrich) (10 μM) was added 12 h before *L. infantum* infection.

2.4. Cytokine Release. To assess the influence of LTB_4 on cytokine production, the liver tissue samples were harvested by a tissue trimmer, weighed, and tittered in 0.5 mL of PBS Complete (Roche Diagnostics, Mannheim, Germany) containing protease inhibitor cocktail. The levels of IFN-γ, IL-17, TNF-α, IL-12p40, IL-23, IL-6, TGF-β, and IL-1β were determined using commercial ELISA kits.

2.5. Cell Culture and Inflammatory Cells Phenotype. Single-cell suspensions of spleen tissue samples from the 5-$LO^{-/-}$ or WT mice at 6th wpi were aseptically prepared, diluted to a concentration of 2×10^6 cells/mL, and dispensed into 48-well plates in a total volume of 500 μL of complete RPMI-1640 medium (1×10^6 cells/well; Gibco) with or without soluble *Leishmania* Ag (5 μg/mL). The cell culture supernatants were harvested after 72 h of culture at 37°C in 5% CO_2, and the cytokine levels in the supernatants were determined by ELISA with commercial kits (BD Biosciences and R&D Systems). For the leukocyte identification, the inflammatory cells were gated based on their characteristic size (FSC) and granularity (SSC), and the T lymphocytes ($CD4^+CD3^+$), dendritic cell activation markers ($CD11c^{high}CD40^+$, $CD11c^{high}CD86^+$, and $CD11c^{high}$MHC-II^+), and neutrophils subsets: activated ($Ly6G^{high}CD11b^{high}$) or inactivated neutrophils ($Ly6G^{int}CD11b^{int}$) were identified individually. For the intracellular staining, the cells were previously cultured with PMA (50 ng/mL) and ionomycin for 4 h in order to obtain the maximum of cytokine production and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's guidelines and stained with anti-IFN-γ or anti-IL-17 conjugated to APC-Cy7 and Alexa700 and with anti-CD3 and anti-CD4 for surface staining with FITC and PerCP, respectively. Rat IgG2b and rat IgG2a were used as the isotype controls. All the antibodies were supplied from BD Biosciences and eBiosciences (San Diego, CA, USA). The cell acquisition was performed using a FACSort flow cytometer. The data were plotted and analyzed using the FlowJo software (Tree Star, Ashland, OR). The total leucocytes counts were determined by relative expression

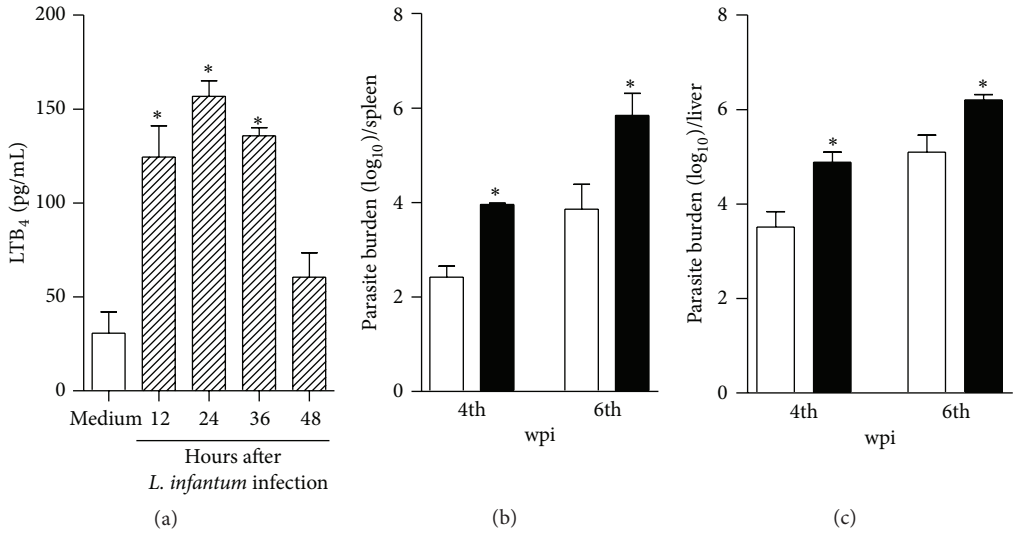

Figure 1: 5-LO activity participates in the control of *L. infantum* infection. In (a), the WT BMDCs (1×10^6 cells/mL) were infected with *L. infantum* (1 : 5) (hatched bar) or medium (white bar) for 12, 24, 36, and 48 h and the LTB_4 amount in the supernatant was determined by ELISA assay. The parasite burden in the spleen (b) and liver (c) was determined in the WT (white bars) and 5-$LO^{-/-}$ (black bars) mice at the 4th and 6th wpi with *L. infantum* promastigote forms (1×10^7 parasites/mice-i.v. route). The data are expressed as the mean ± SEM, $N = 5$-6. $^*P < 0.05$ compared with the WT group.

of leucocytes subpopulation stained with specific antibody obtained in 300,000 events acquired and proportional to the leukocytes number obtained in Neubauer chamber.

2.6. Statistical Analysis. The data are expressed as the mean ± SEM and are representative of 2–4 independent experiments. The results from the individual experiments were not combined because they were analyzed individually. The means from the groups were compared by ANOVA followed by Tukey's honest significant difference (HSD) test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. 5-LO Activation Is Required for Experimental L. infantum Infection Control. To determine whether *Leishmania infantum* drives the activation of 5-LO pathway, we performed a kinetic in the release of LTB_4 by bone marrow-derived dendritic cells after 12, 24, 36, or 48 hours of parasite infection. *L. infantum* induces significant amounts of LTB_4 by BMDCs at 12 hours postinfection, peaked at 24 hours, and persisted for 36 hours. At 48 hours, the heightened levels of LTB_4 production were significantly reduced and similar that produced for uninfected cells (medium stimuli) (Figure 1(a)). To characterize the LTB_4 function, 5-$LO^{-/-}$ and littermate control mice were infected with *L. infantum* and the course of infection was monitored by parasite quantification into the organs by a limiting dilution. We observed that WT presented progressive parasite titers into the spleen (Figure 1(b)) and the liver (Figure 1(c)) over time. The 5-$LO^{-/-}$ mice were more susceptible to infection, harboring more parasites in both target organs than were the WT animals in all the analyzed periods, demonstrating that 5-LO activity, and possibly LTB_4, participates in the control of *L. infantum*.

3.2. L. infantum-Infected 5-$LO^{-/-}$ Mice Fail to Recruit Neutrophils to Infectious Foci. Because LTB_4 presented potent neutrophil chemotactic activity and we and others reported the role of neutrophils in the control of *Leishmania spp.* [11], we characterized the neutrophils present in the spleens of the 5-$LO^{-/-}$ or WT infected mice at 6th wpi.

Based on their characteristic size (FSC) and granularity (SSC), we observed a significant reduction of cells when analyzed in the granulocytes gate from infected 5-$LO^{-/-}$ mice. Phenotyping the cells, we found that the frequencies of $Ly6G^+CD11b^{high}$ were present in the spleen samples from the WT infected mice. The percentage of the influx of neutrophils was affected in the 5-$LO^{-/-}$ infected mice, which showed an approximately 30% reduction compared with that of the WT infected mice (Figures 2(b) and 2(c)). In terms of total numbers, the neutrophil reduction was ~50% in the 5-$LO^{-/-}$ mice (Figure 2(c)). We also observed another neutrophils population, $LY6G^+CD11b^{interm}$ (Figure 2(b)), features of inactive neutrophils since CD11b is upregulated under proinflammatory stimuli [31, 32]. However, their frequency and total cells (Figure 2(d)) in the spleens were similar in both groups. These findings suggest that 5-LO activity participates in neutrophil recruitment to inflammatory foci and, under appropriated activation, might be required for parasite control during *L. infantum* infection.

3.3. 5-LO Activity Is Associated with the Development of Host Protective Th17 Responses. Because the development of IFN-γ and IL-17-producing $CD4^+$ T helper cells is crucial

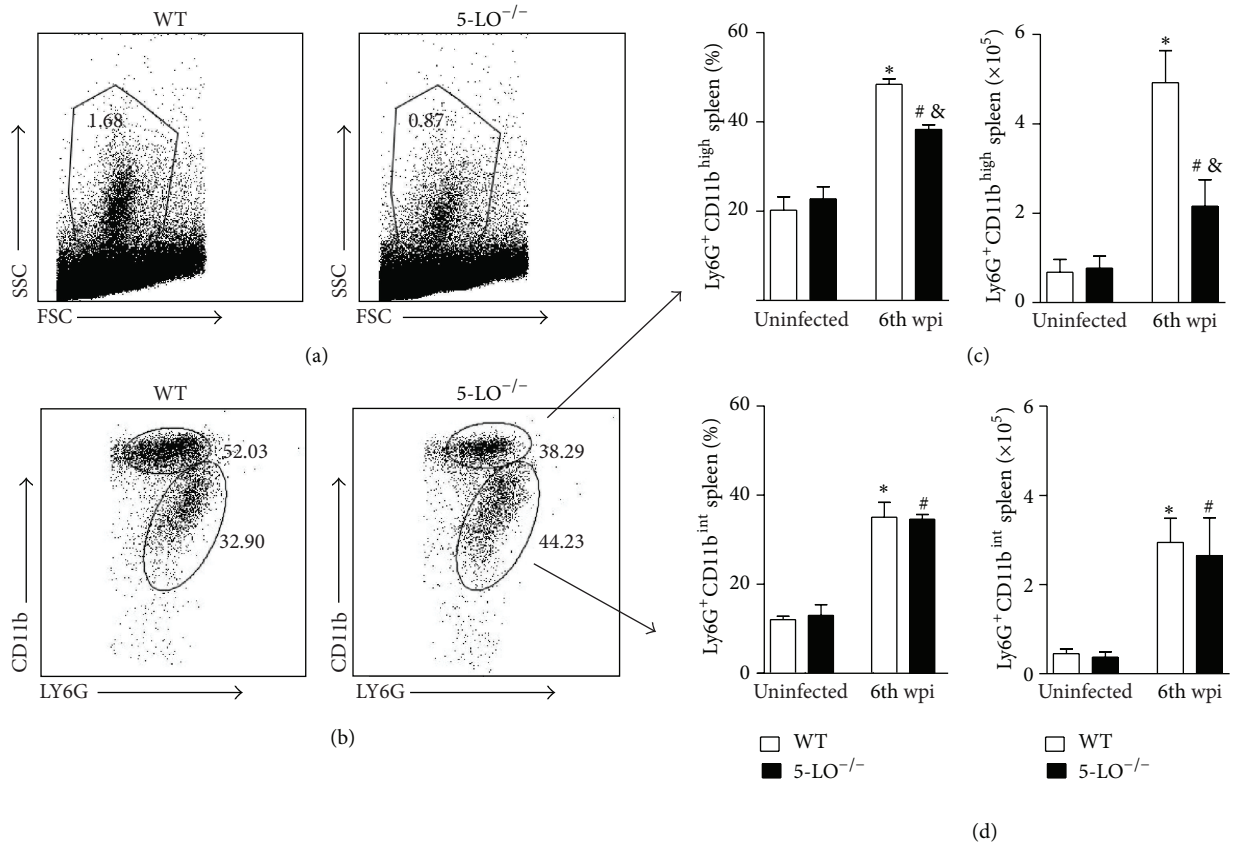

Figure 2: Lack of 5-LO interferes on neutrophil migration. The neutrophils were gated based on their characteristic size (FSC) and granularity (SSC) (a). The dot plots represent the frequency of neutrophils population characterized by $LY6G^{high}CD11b^{high}$ (upper gate) and $LY6G^{int}CD11b^{int}$ (lower gate) by flow cytometry (b). The bar graphs display the percentage and the absolute number characterized as $LY6G^{high}CD11b^{high}$ population (c) or $LY6G^{int}CD11b^{int}$ (d) in the spleen from the WT and 5-$LO^{-/-}$ mice at the 6th wpi or from uninfected mice (naïve mice). The data are expressed as the mean ± SEM, $N = 5$-6. $^{*}P < 0.05$ compared with the WT naïve group, $^{\&}P < 0.05$ compared with the WT infected group, and $^{\#}P < 0.05$ compared with 5-$LO^{-/-}$ naïve group.

for the control of parasite replication in the target organs of LV, we investigated whether these responses were generated in a 5-LO dependent manner. Spleen cells from WT and 5-$LO^{-/-}$ mice at 6th wpi or naïve were *in vitro* restimulated with polyclonal PMA plus ionomycin and the intracellular cytokine production was analyzed. There was no difference in the frequency and absolute number of the IFN-γ-producing $CD4^+$ T cells in the WT and 5-$LO^{-/-}$ mice (Figure 3(a)). The IL-17-producing $CD4^+$ T cells were significantly impaired in the spleens of the 5-$LO^{-/-}$ mice (Figure 3(b)), where the Th17 cells reduction was approximately 50% of that in the WT mice.

Having determined that 5-LO activity participates in the development of the Th17 response, we measured the production of cytokines in the culture supernatant of the total splenic cells from the WT, 5-$LO^{-/-}$ naïve, or infected mice at 6th wpi and restimulated them *in vitro* with soluble *Leishmania* Ag (SLA). The stimulation with SLA did not induce significant amounts of IFN-γ (Figure 4(a)), IL-17 (Figure 4(b)), TNF-α (Figure 4(c)), IL-23 (Figure 4(d)), IL-6 (Figure 4(e)), IL-1β (Figure 4(f)), and TGF-β (Figure 4(g)) in the culture supernatants of spleens cells from the naïve WT mice compared with those induced in the control (medium). A similar effect was observed in cells from the 5-$LO^{-/-}$ naïve mice when stimulated with the antigen. The infection promoted pronounced levels of all the analyzed cytokines after the SLA stimulation in the WT group, compared to those in the medium (Figures 4(a)–4(g)). Infection in the 5-$LO^{-/-}$ mice resulted in a reduction of cytokine release related to the Th17 axis such as IL-17, TNF-α, IL-23, and IL-6 (Figures 4(b)–4(e)); however, neither IFN-γ (Figure 4(a)), IL-1β (Figure 4(f)), nor TGF-β (Figure 4(g)) productions were affected by a specific stimulus, compared to that obtained in the infected WT mice when stimulated with SLA. Additionally, proinflammatory cytokines in the liver involved in the Th17 axis such as IL-17 (Figure 4(i)), TNF (Figure 4(j)), and IL-12p40 (IL-23) (Figure 4(k)) were reduced in the absence of 5-LO. Corroborating to Figures 3(a) and 4(a), IFN-γ amounts were not altered in the deficient mice (Figure 4(h)). These data suggest that 5-LO activity is associated with Th17 response development, and this pathway might be involved in the neutrophils recruitment to inflammatory foci.

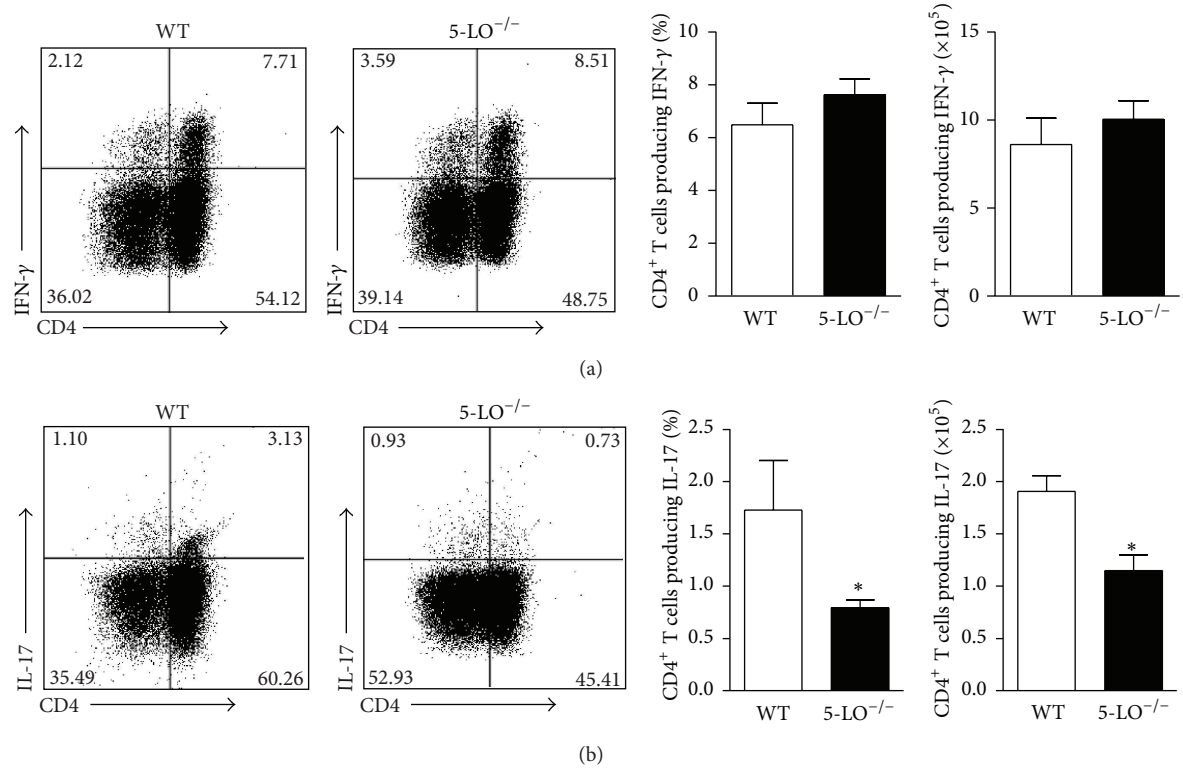

FIGURE 3: 5-LO ablation decreased the Th17, but not the Th1, pattern of immune response. The spleen cells from the WT (white bars) or 5-LO$^{-/-}$ (black bars) mice at the 6th wpi were *in vitro* restimulated with PMA and ionomycin for 4 h and analyzed for intracellular cytokine production by flow cytometry. The dot plots represent the frequency of the CD4$^+$ T cell-producing IFN-γ (a) and the CD4$^+$ T cell-producing IL-17 (b), and the graph bars represent the percentage and the total number of these cells. The data are expressed as the mean ± SEM, N = 5-6. $^*P < 0.05$ compared with the WT group.

3.4. DCs Activation May Be Related to 5-LO Activity during Parasite Infection. Because dendritic cells (DCs) are the main cells involved in orchestrating immune responses during *Leishmania sp.* infection through the release of cytokines which might be involved in the differentiation of Th17 cells [33], we first evaluated, using flow cytometry analyses, the maturation profile of dendritic splenic cells of the WT and 5-LO$^{-/-}$ mice infected at 6th wpi by evaluating the costimulatory molecules in the CD11c^{high} cells. In terms of percentage, the DC expressing CD86 (Figure 5(b)) or MHC-II (Figure 5(c)) is slightly reduced in the absence of 5-LO that was ~20% less compared with WT. However, in terms of total cells, we observed a markedly reduction of DCs expressing CD86 or MHC-II that was ~50% into spleens of 5-LO$^{-/-}$ mice (Figures 5(b)-5(c), resp.). No difference was observed in the CD40 expression (Figure 5(a)). Consistent with the *in vivo* data, the bone marrow-derived DC (BMDC) from the WT infected *in vitro* with parasites enhanced the expression levels of surface markers such as MHC-II, CD40, and CD86 (Figures 6(a)–6(c)), when compared to those of the medium. In contrast, infection of BMDC from 5-LO$^{-/-}$ with *L. infantum* inhibits their activation, presenting reduced expression of CD86 surface markers (Figure 6(c)). The absence of 5-LO did not alter the LPS-induced dendritic cell maturation (Figures 6(a)–6(c)).

Next, we evaluated the release of innate cytokines involved in Th17 axis differentiation by DCs. Thus, we determined the levels of TNF, IL-23, IL-1β, and IL-6 in the supernatants from the WT or 5-LO$^{-/-}$ BMDCs cultured with *L. infantum* parasites or medium. As the positive control, the cells were activated with LPS. The parasites induced significant production of TNF (Figure 6(d)), IL-23 (Figure 6(e)), IL-1β (Figure 6(f)), and IL-6 (Figure 6(g)) by the DC from WT when compared with that of the respective control group. Additionally, the parasites promoted significant amounts of cytokines in the DC from 5-LO$^{-/-}$, compared to the 5-LO$^{-/-}$ DC stimulated with the medium; however TNF (Figure 6(d)), IL-23 (Figure 6(e)), and IL-6 (Figure 6(g)) levels were significantly decreased comparing those released by infected WT DC. The levels of IL-1β (Figure 6(f)) were unaltered in the absence of 5-LO. These data suggest that 5-LO participates in DC activation, interfering with the cytokine release involved in the Th17 subset polarization during an experimental *L. infantum* infection.

The ablation of 5-LO lacks not only LTB_4, but also cysteinyl leukotrienes including LTC_4, LTD_4, and LTE_4 activity [34]. In other to clarify, in part, the effect of LTB_4 during *L. infantum* infection, we use *in vitro* a selective BLT_1 leukotriene B_4 receptor antagonist (U-75302). The BLT_1 antagonist was added to BMDC culture 12 h

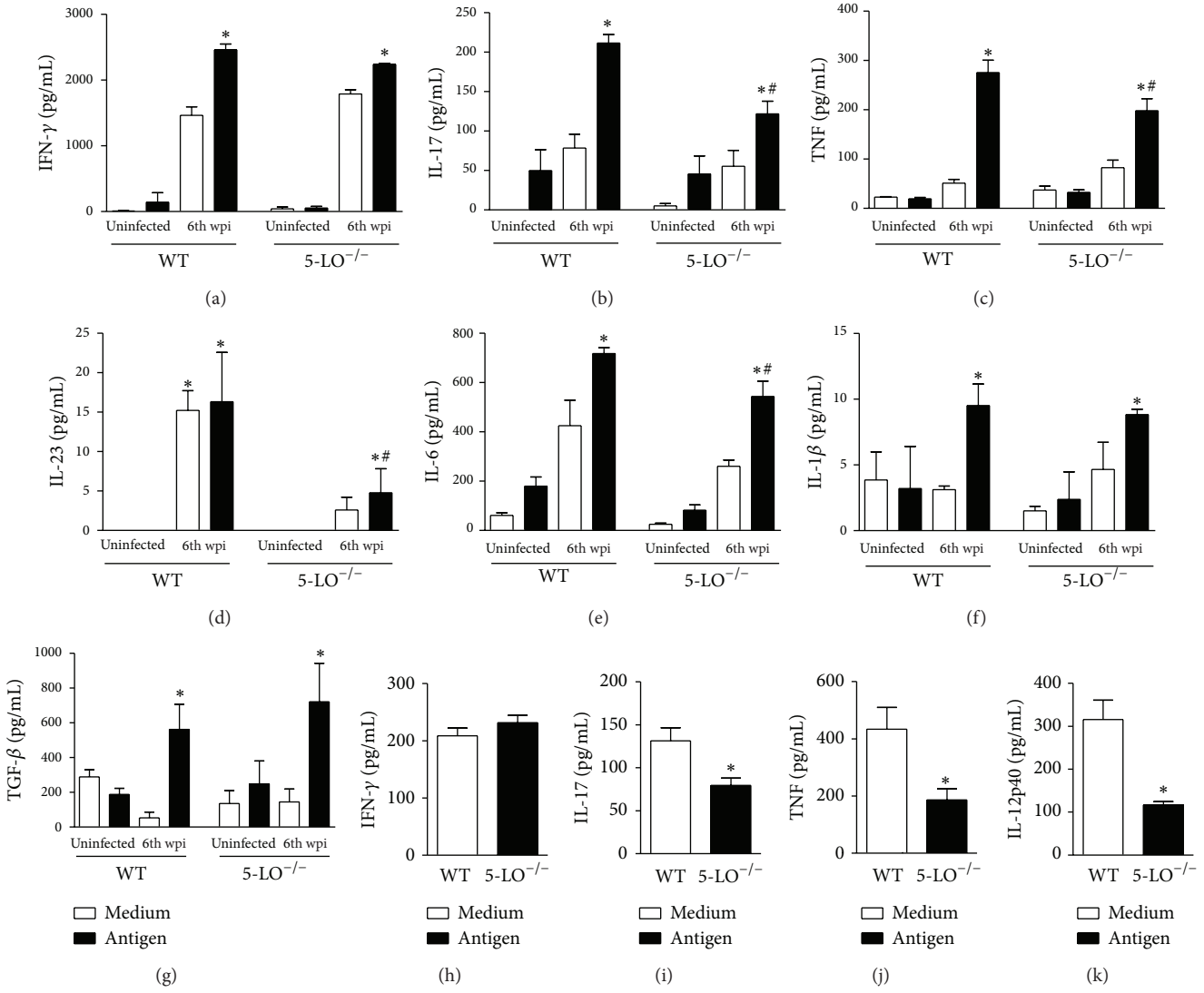

Figure 4: The absence of 5-LO affected the cytokine release related to Th17 pattern. The spleen cells from the WT and 5-LO$^{-/-}$ mice at the 6th week pi or uninfected were *in vitro* stimulated with the *L. infantum* antigen (50 μg/mL) or medium for 72 hours, and the levels of IFN-γ (a), IL-17 (b), TNF (c), IL-23 (d), IL-6 (e), IL-1β (f), and TGF-β (g) were measured in the culture supernatants by ELISA assay. The data are expressed as the mean ± SEM and one representative of two independent experiments. $^{*}P < 0.05$ compared to the medium; $^{\#}P < 0.05$ compared with the WT stimulation. The liver fragments from the WT (white bar) or 5-LO$^{-/-}$ (black bar) at the 6th wpi with the *L. infantum* promastigote forms were collected and weighed for the determination of IFN-γ (h), IL-17 (i), TNF (j), and IL-12p40 (k) by ELISA in the homogenate supernatants. The data are expressed as the mean ± SEM, $N = 5$-6. $^{*}P < 0.05$ compared with the WT group.

before *L. infantum* infection and the release of cytokines related to Th17 pattern was measured into culture supernatant by ELISA assay. As expected, TNF (Figure 6(h)), IL-23 (Figure 6(i)), and IL-6 (Figure 6(j)) were produced during infection. The ability of BMDCs infected with parasites to produce cytokines such as TNF (Figure 6(h)) and IL-23 (Figure 6(i)), but not IL-6 (Figure 6(j)), was inhibited by BLT_1 blockage, confirming that LTB_4 is associated with the release of cytokines involved in the Th17 axis. We do not rule out the possibility of others leukotrienes that may contribute to cytokine release, herein, that is, IL-6 release, but we undoubtedly evidenced LTB_4 participation in the control of VL.

4. Discussion

In this study, we report 5-LO activity, and presumably LTB_4, as an important mediator in controlling infection induced by *Leishmania infantum*. This eicosanoid that is released during infection may promote the activation of dendritic cells, which influence the release of mediators involved in the drive of naive $CD4^{+}$ T lymphocytes to the Th17 profile. In the last instance, the Th17 subtype recruits neutrophils to the infection foci that might retrain the parasite restriction. Understanding the role of LTB_4 in the inflammatory process mediated by *L. infantum* might elucidate some of the effector mechanisms that control the replication of the parasites.

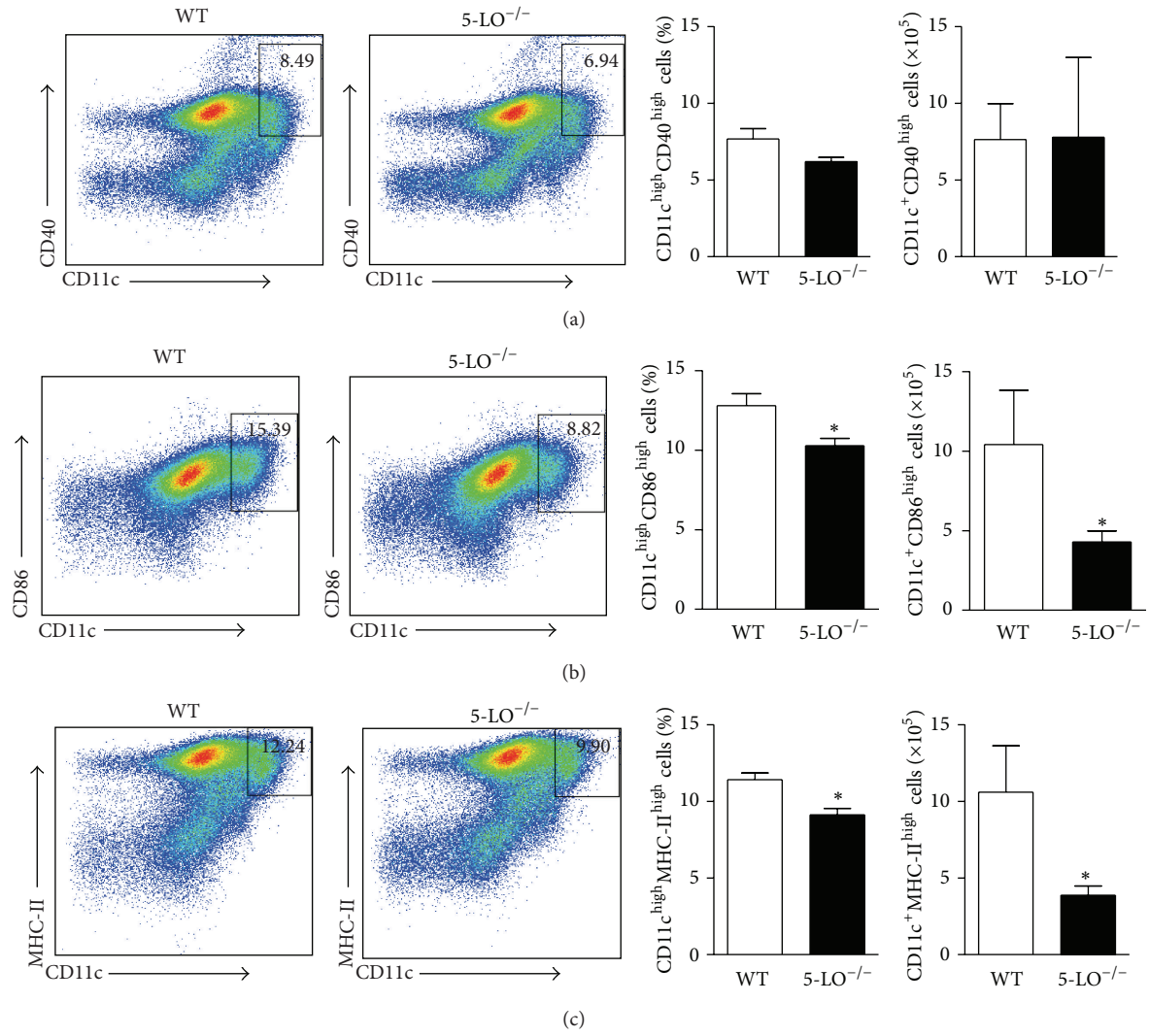

FIGURE 5: 5-LO activity is required for dendritic cell activation into the inflammation site. The *in vivo* surface markers of DCs from the WT (white bars) or 5-LO$^{-/-}$ (black bars) infected mice at the 6th wpi were determined by flow cytometry. The dot plots represent the frequency of CD40 (a), CD86 (b), and MHC-II (c) in the CD11c^{high} population. All analyses were performed on CD11b^{+}CD11c^{high} gated cells. The data are expressed as the mean ± SEM, $N = 5$-6. $^{*}P < 0.05$ compared to the WT group.

We demonstrated that infection with *L. infantum* results in the production of LTB_4 by dendritic cells during *in vitro* infection. The absence of endogenous LTB_4 promoted higher susceptibility to infection. The genetic ablation of 5-LO harbored more parasites in target organs such as the spleen and liver, demonstrating its role in the control of infection. These results are consistent with those of previous studies that demonstrate the role of LTB_4 in the control of infectious processes [13, 17, 21], increasing the leishmanicidal activity of macrophages [24] and of neutrophils [25] by a nitric oxide (NO)-dependent mechanism and release of reactive oxygen species (ROS), respectively.

Several studies have demonstrated that LTB_4 is a potent inducer of neutrophils. During leishmaniasis, neutrophils are rapidly mobilized to the inflammatory site [1, 35], where they eliminate the pathogen by the production of reactive oxygen species (ROS) and the release of peptides and antimicrobial proteases [36–38]. In our results, the high susceptibility observed in animal 5-LO$^{-/-}$ was accompanied by the failure of neutrophil migration. LTB_4 has a central role in controlling the migration of neutrophils to sites of inflammation through BLT_1 and BLT_2 (leukotriene receptors) [39], directly by inducing the expression of the CD11b and CD18 integrins [40] or indirectly by amplifying the production of inflammatory mediators such as cytokines and chemokines by others cells [41, 42]. In fact, we observed a significant reduction of activated neutrophils expressing CD11b into target organs that were infected by parasites in the absence of 5-LO. Furthermore, LTB_4 enhances effectors mechanisms of neutrophils such as phagocytic capacity [43] and granules releasing and stimulates the enzymatic generation of ROS [14, 44, 45], including *in vitro* infection by *L. amazonensis* [25].

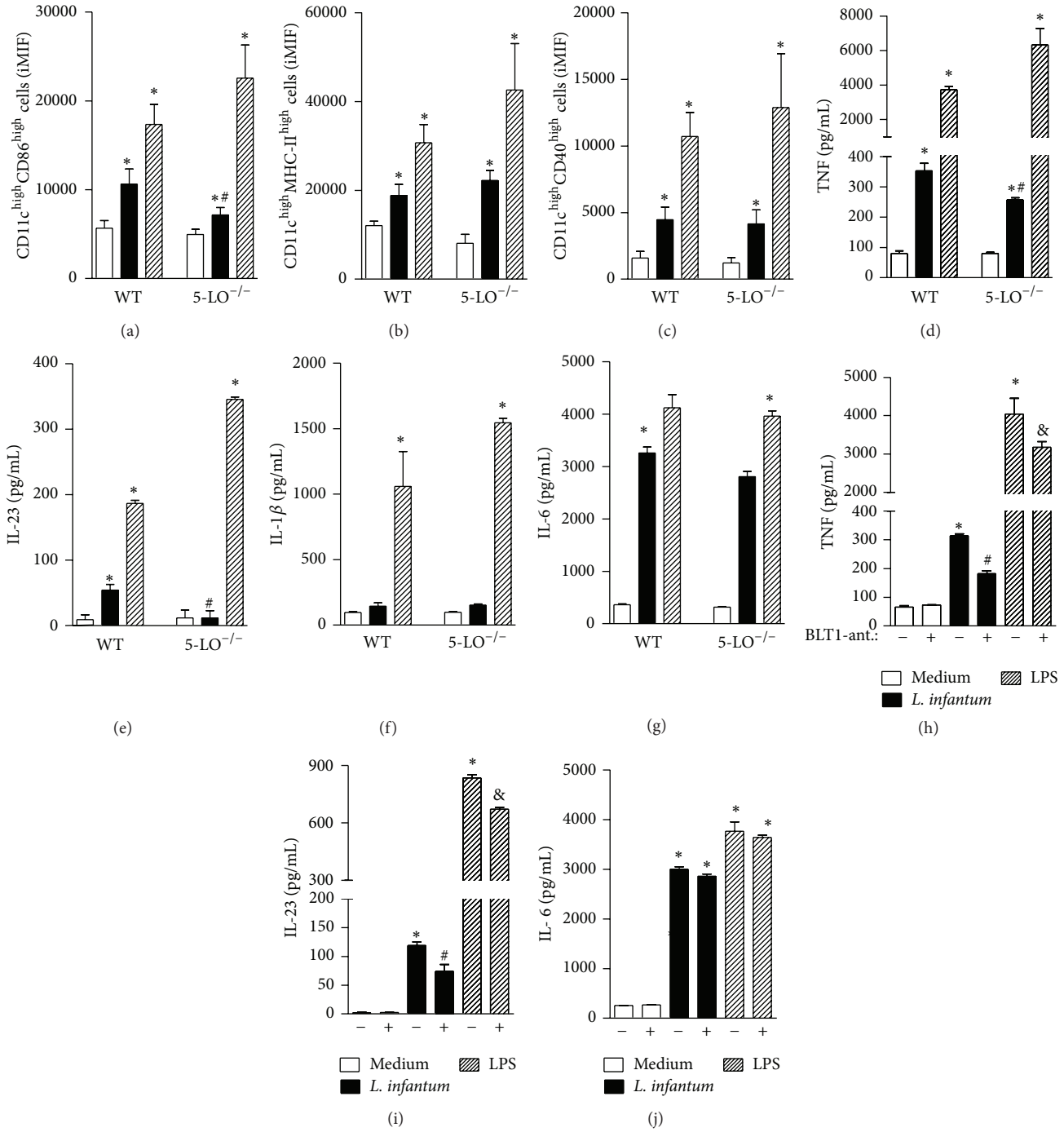

FIGURE 6: The absence of 5-LO interferes with BMDC maturation and the release of innate cytokines induced by *L. infantum* through BLT_1 receptor. The WT or 5-$LO^{-/-}$ BMDC was stimulated with *L. infantum* (5 : 1) (black bars), LPS (200 ng/mL) (hatched bars), or medium (white bars) for 24 h. The BMDCs were harvested and the costimulatory molecules expression such CD86 (a), MHC-II (b), and CD40 (c) was evaluated by flow cytometry. All analyses were performed for the $CD11c^{high}$ population. The TNF (d), IL-23 (e), IL-1β (f), and IL-6 (g) levels were measured in the supernatant of the BMDC culture by ELISA assay. The data are expressed as the mean ± SEM and are representative of three independent experiments. $^{*}P < 0.05$ compared with the medium; $^{\#}P < 0.05$ compared with the infected WT. $^{\&}P < 0.05$ compared with the infected WT. In some experiment, selective BLT_1 leukotriene B_4 receptor antagonist (10 μM) was or not added 12 h before *L. infantum* infection or LPS stimuli. The levels of TNF (h), IL-23 (i), and IL-6 (j) were determinate into supernatant 24 thereafter. $^{*}P < 0.05$ compared with the medium; $^{\#}P < 0.05$ compared with *L. infantum* infection. $^{\&}P < 0.05$ compared with LPS stimuli.

Thus, it seems that the protector role of LTB_4 during LV may be played by the recruitment and activation of neutrophils to the site of infection.

The recruitment of neutrophils might be induced by cytokines such as IL-17 because they are potent granulopoietic factors [46] that induce the release of CXC chemokines [47]. We found that the absence of LTB_4 synthesis impaired the Th17 response, whereas the Th1 response was unchanged in the target organs. Consistently, the production of IL-17 by spleen cells in response to the specific stimulus (i.e., *Leishmania* antigen) and its detection in the liver of 5-$LO^{-/-}$ infected mice was inhibited, confirming the interference of LTB_4 in the release of IL-17. We have not evaluated whether LTB_4 participates in the control of *Leishmania infantum* through Th17-dependent manner; however, we believe that the administration of recombinant IL-17 may rescue the protective effect of leucotrienes in susceptible 5-LO deficient mice. In fact, administration of recombinant IL-17 or IL-23 in susceptible BALB/c mice infected with *L. donovani* controlled parasite replication, which was associated with increased iNOS activity [3]. Furthermore, exogenous LTB_4 is able to positively modulate the differentiation of Th17 cells from naive $CD4^+$ T cells [48]. The induction of experimental autoimmune encephalomyelitis (EAE) in animals genetically deficient in BLT_1 presented clinical score signs attenuated because of impairment of the Th17 generated response. Infiltration of T cells, macrophages, and granulocytes into the spinal cord was reduced in the $BLT_1{}^{-/-}$ mice [49], demonstrating the involvement of LTB_4 in the development of the Th17 response.

LTB_4 is produced during inflammatory and infectious processes by several leucocytes [50], including activated neutrophils, macrophages, and T cells [51–54]. Among the cells able to synthesize LTB_4, DCs play an important role in the initiation of immune responses because they are the main cells involved in pathogen recognition, triggering several proinflammatory mechanisms that bridge to adaptative immune responses [55–57]. According to our results, DCs are potential sources of LTB_4 during *L. infantum* infection. Given the importance of the role lipid mediators play in leucocyte activation, LTB_4 production by DCs is a major mechanism for the modulation of the effector function of other cell subsets during LV, for example, mediating the recruitment of neutrophils to inflammation sites. We do not rule out the possibility of others leukotrienes that may contribute to cytokine release, since that the ablation of 5-LO lacks not only LTB_4 but also cysteinyl leukotrienes including LTC_4, LTD_4, and LTE_4 activity [34]. However, the pharmacological blockage of BLT_1 prevented, at least in part, the release of cytokines by DC, evidencing LTB_4 association with Th17 axis, and in last instance, controlling parasite replication.

Apart from sources of LTB_4, DCs are the target of the action of lipid mediators as an important mechanism for modulating the immune response [58, 59]. An impaired Th17 response might result from failed DC activation in the absence of 5-LO. This hypothesis might be supported by the following explanations. First, exploring the role of LTB_4 in DC activation, our data demonstrated that the maturation phenotype of DCs from animal 5-$LO^{-/-}$ was reduced during *in vivo* and *in vitro* infection. Consistently, the addition of LTB_4 in cultured BMDCs induces maturation of these cells to increase MHC-II expression. Blockage of 5-LO with NDGA protects cells from the effects of LTB_4 on DC maturation [60]. BMDCs migrate and are activated in response to LTB_4, and its effect is lost in cells that lack BLT_1 [61]. LTB_4 upregulates the expression of CCR7 and its ligand CCL19/ELC, which mediate the migration to lymphoid organs. Second, the impaired ability of DCs from animal 5-$LO^{-/-}$ to secrete cytokines is involved in the polarization of naïve CD4 T cells to the Th17 profile. Naïve $CD4^+$ T lymphocytes are polarized to the Th17 subset through the combined pattern of the action of cytokines such as IL-1β, TGF-β and IL-6 [62], whereas activation requires sustained stimulation with IL-23, which is predominantly produced by dendritic cells and TNF release [63]. Our data demonstrated that the production of TNF, IL-23, and IL-6 *in vivo,* at least, was compromised in the absence of 5-LO. Supporting our hypothesis, a significant reduction of IL-23, TNF, and IL-6 by BMDC was observed in the dendritic cells derived from animal 5-$LO^{-/-}$. Consistently, Lefèvre and colleagues demonstrated that cytokines such as IL-1β, TGF-β, and IL-6 are highly produced by macrophages infected *in vitro* with *L. infantum* [29]. The role of LTB_4 in the induction of innate cytokines related to the Th17 profile differentiation released by DCs is unprecedented. It is known that IL-1R signaling is dependent on the BLT_1 downstream pathway. The requirement for the BLT_1 signaling pathway is overcome by exogenous administration of IL-1β in $LTB_4{}^{-/-}$ mice [64]. Moreover, BLT_1 expression is upregulated in Th17-differentiated T cells [49] and *ex vivo* studies have demonstrated that the production of TNF and IL-6 was impaired in the absence of $BLT_1{}^{-/-}$ cells [65, 66], confirming the role of LTB_4 in driving the Th17 response.

We do not investigate the molecular mechanisms by which 5-LO activity interferes with maturation process and subsequent activation of dendritic cells, but we believe that the initial response is dependent on TLR4 signaling. During parasite recognition through TLR4 pathway, the adapter molecule MyD88 is recruited and activates factors such as NF-κB [67, 68], leading to transcription of proinflammatory cytokines such as TNF, IL-6, and IL-23. MYD88 recruitment also activated 5-LO enzyme, promoting the synthesis of leucotriens, especially LTB_4 that, through BLT_1 pathway, amplifies the activation of NF-κB which may induce cellular activation [66]. Interestingly, genetic deletion of 5-LO or pharmacological blockade of BLT_1 receptor interferes with the secretion of proinflammatory cytokines by DCs and their maturation phenotype. The 5-LO pathway may act in autocrine manner, increasing the activation and function of DCs, and greatly influence the magnitude response of Th17 cells as well. Thus, the amplification of the inflammatory response mediated by 5-LO activation during parasite recognition by DCs appears to play an important role in controlling parasite replication.

5. Conclusion

Our data demonstrated that 5-LO activity, and perhaps LTB_4, plays a prominent role in controlling *L. infantum*-induced visceral leishmaniasis, which may be associated with the development of the Th17 response and the subsequent recruitment of neutrophils to the inflammatory site that is dependent on dendritic cell activation. Future studies might characterize which innate receptors on DCs are involved in the recognition of the parasite, leading to a subsequent synthesis of LTB_4. The results show, for the first time, the role of LTB_4 in the development of the Th17 response in the context of an infectious disease.

Conflict of Interests

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

Acknowledgments

The authors are grateful to Giuliana Bertozi for helping with the LTB_4 measurement. The research leading to these results received funding from the CAPES-PRODOC, São Paulo Research Foundation (FAPESP) under Grant Agreements nos. 2012/14524-9 (Thematic Project) and 2013/08216-2 (Center for Research in Inflammatory Disease) and from the University of São Paulo NAP-DIN under Grant Agreement no. 11.1.21625.01.0.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 316204, 9 pages
http://dx.doi.org/10.1155/2014/316204

Review Article

Neuroprotective Effects of Lipoxin A4 in Central Nervous System Pathologies

Alessandra Cadete Martini,[1] Stefânia Forner,[1] Allisson Freire Bento,[2] and Giles Alexander Rae[1]

[1] *Departmento de Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina (UFSC), Campus Universitário, Trindade, 88049-900 Florianópolis, SC, Brazil*
[2] *Centro de Inovação e Ensaios Pré-Clínicos (CIEnP), Av. Luiz Boiteux Piazza, 1302-Canasvieiras, 88056-000 Florianópolis, SC, Brazil*

Correspondence should be addressed to Alessandra Cadete Martini; alessandracmartini@gmail.com

Received 19 June 2014; Accepted 12 August 2014; Published 9 September 2014

Academic Editor: Alexandre de Paula Rogerio

Many diseases of the central nervous system are characterized and sometimes worsened by an intense inflammatory response in the affected tissue. It is now accepted that resolution of inflammation is an active process mediated by a group of mediators that can act in synchrony to switch the phenotype of cells, from a proinflammatory one to another that favors the return to homeostasis. This new genus of proresolving mediators includes resolvins, protectins, maresins, and lipoxins, the first to be discovered. In this short review we provide an overview of current knowledge into the cellular and molecular interactions of lipoxins in diseases of the central nervous system in which they appear to facilitate the resolution of inflammation, thus exerting a neuroprotective action.

1. Introduction

Neurological diseases, such as Alzheimer's disease, Parkinson's disease, traumatic brain injury, and stroke, among others, as well as conditions leading to chronic neuropathic pain, typically present marked transient or continued neuroinflammation. Whether this inflammatory state has beneficial or detrimental effects is still controversial. Orchestrated actions of microglia, macrophages, and lymphocytes result in a protective mechanism to isolate the damaged brain tissue and destroy the affected cells. Thus, inflammatory responses generally result in a self-limiting healing process. However, if this response is not adequately controlled, the immune system begins to attack previously undamaged cells, which may cause a progressive neuronal loss, amongst many other detrimental effects [1].

Many studies have raised the question that the beneficial effects of diet supplementation with omega-3 (ω-3) polyunsaturated fatty acids (PUFAs) could be the result of their metabolism into potentially anti-inflammatory substances [2–5]. Indeed, a growing body of evidence indicates that inflammation may be modulated by endogenously produced lipids that actively participate in dampening host responses to injury, leading to active resolution of the inflammatory process [6]. This group of endogenous proresolving lipid mediators currently comprises lipoxins (LXs), resolvins, protectins, and maresins, all of which have the potential to actively resolve inflammation by signaling metabolic, cellular, and tissue events to return to homeostasis after inflammation, in a process known as catabasis [7].

All known proresolving lipid mediators are synthetized from PUFAs. Whereas the starting point for synthesis of LXs is arachidonic acid (AA), a ω-6 PUFA generated from linoleic acid, resolvins and protectins are products originated from the ω-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively [8]. Indeed, the same enzymes that metabolize linoleic acid to AA can also convert α-linoleic acid into EPA and DHA. However, as the proportion of AA in inflammatory cell membranes is much higher than those of ω-3 PUFAs, substrate availability for

metabolism of AA by cyclooxygenase (COX) or lipoxygenase (LOX) isozymes is far greater than is seen regarding the metabolism of EPA and DHA. The only two truly endogenous LXs known, LXA4 and LXB4, are typically formed by transcellular metabolism of AA involving sequential LOX activity [9]. In one of these pathways, AA is oxygenated by 15-LOX to generate 15S-HETE, which is then modified by 5-LOX to originate both LXs. Another 2-step pathway for LXA4 and LXB4 formation involves the conversion of AA into leukotriene A4 by LOX-5, followed by its metabolism by LOX-12 [7]. Interestingly, the acetylation of COX-2 by aspirin, while inhibiting the synthesis of prostaglandins and thromboxane, favors the generation of 15R-HETE, which can then be converted by LOX-5 to generate the aspirin-triggered LXs (ATLs) 15-epi-lipoxin A4 and 15-epi-lipoxin B4 [10]. The LXs are subjected to rapid enzymatic breakdown, but ATLs are more resistant to degradation and thus can exert longer-lasting effects. The synthetic pathways of proresolving lipid mediators are depicted in Figure 1, but further details on the synthesis and biological effects of resolvins, protectins, and maresins can be found elsewhere [3, 11, 12].

LXs (and ATLs) promote the majority of their effects by acting on a specific G protein-coupled receptor designated as the ALX/FPR2 receptor, a member of the formyl peptide receptor superfamily. This receptor is found in a wide array of tissues, including spleen and lungs, and cells such as macrophages, neutrophils, and microglia and is coupled to various specific signaling pathways, depending on where they are expressed [13]. The ALX/FPR2 receptor also responds to resolvins and several peptides, some of which, like annexin-1, are proresolving, while others, such as amyloidogenic peptides, are proinflammatory [14]. Importantly, LXA4 can also bind to additional receptors, including the aryl hydrocarbon receptor AhR [15], the cysteinyl leukotriene receptor (CysLT) [16, 17], the GPR32 receptor [18], and the CB1 cannabinoid receptor [19]. However, LXA4 does not always act as an agonist when bound to these receptors, as it is a partial antagonist of the CysLT receptor [14] and an allosteric signaling enhancer at CB1 cannabinoid receptors [19].

Although LXs are AA-derived eicosanoids, they can be clearly distinguished from the classical proinflammatory prostaglandins, thromboxane, and leukotrienes on the basis of their capacity to trigger a self-limiting response to inflammation when generated by leukocytes. In fact, their formation and functions are directly linked to a change in the phenotype of neutrophils present at the site of inflammation [20]. Once formed at the site of injury, LXs suppress neutrophil recruitment, enhance phagocytosis of apoptotic neutrophils by macrophages, and stimulate the accumulation of a nonphlogistic type of monocytes/macrophages which do not produce proinflammatory mediators [21].

A growing number of studies have demonstrated the roles of LXs as anti-inflammatory and proresolving agents in different animal models of peripheral and central disorders, including cardiovascular diseases, as reviewed by others [6, 10, 22, 23]. Here we will specifically provide an overview of the profile of biological actions of LXs that might be relevant to their potential use as therapeutic agents for inflammatory disorders in the central nervous system (CNS).

2. Alzheimer's Disease

Alzheimer's disease (AD) is a devastating neurodegenerative syndrome characterized by drastic and progressive dementia and changes in behavior, allied to accumulation in the brain of extracellular senile plaques composed mainly of amyloid β protein (Aβ), intraneuronal neurofibrillary tangles containing hyperphosphorylated tau protein, and chronic neuroinflammation. This disease affects millions of people worldwide, especially late in life, and its causes are incompletely understood [24]. Despite intense efforts, at present AD has no cure and available supportive treatment is far from being efficient. This, in association with the marked increase in life expectancy of the world population, renders the search for more effective treatments of AD one of the greatest challenges in modern medicine.

The role of lipids in AD pathogenesis has been analyzed by several groups, and some studies showed that brains of patients with AD present possible aberrant lipid metabolism [25–27]. The neurodegenerative process in AD is closely related with an inflammatory response in the brain which involves several AA-derived lipid inflammatory mediators [28]. Indeed, a very recent study has revealed that the resolution of inflammation is impaired in the brain of AD patients [29]. The study found that LXA4 levels in post-mortem samples of cerebrospinal fluid and hippocampus of AD patients were lower than those of control subjects and that this decrease was correlated with the degree of cognitive deficit and tissue accumulation of tau protein. Conversely, the expression of ALX/FPR2 receptors was clearly greater in AD hippocampal samples.

Intriguingly, amyloid β protein (Aβ), one of the major contributors to AD pathogenesis, binds to and activates ALX/FPR2 receptors, but with antagonistic effects [30]. Le and colleagues [30] showed that $A\beta_{1-42}$ exerts chemotactic activity in human leucocytes through ALX/FPR2 receptor activation. Accordingly, another study showed that Aβ, acting via ALX/FPR2 receptors, induces chemotaxis and superoxide production in mouse neutrophils and stimulates cultured murine microglial cells, which strongly suggested its pivotal role in recruitment of microglial cells to senile plaques, induction of oxidative stress, and consequent neuroinflammation in AD [31]. These and other experimental observations clearly establish ALX/FPR2 receptors as pathophysiologically relevant in Aβ-mediated proinflammatory responses in AD [32].

On the other hand, a recent study observed that prolonged twice-daily treatment with the ATL 15-epi-lipoxin A4 (ATLA4) promoted impressive effects in a genetically based murine model of AD [33]. Among the more outstanding findings of the study, ATLA4 downregulated brain production of the proinflammatory mediators TNF-α, interleukin-1β (IL-1β), interferon-γ, IL-6, GM-CSF, and RANTES and of MMP-9, all of which are strongly related to AD progression. Conversely, ATLA4 increased brain levels of the anti-inflammatory cytokines IL-10 and TGF-β, stimulated the accumulation of alternative microglial cells which, unlike the classical ones, display a nonphlogistic phenotype, and enhanced the clearance of Aβ in CNS. Of note, and in

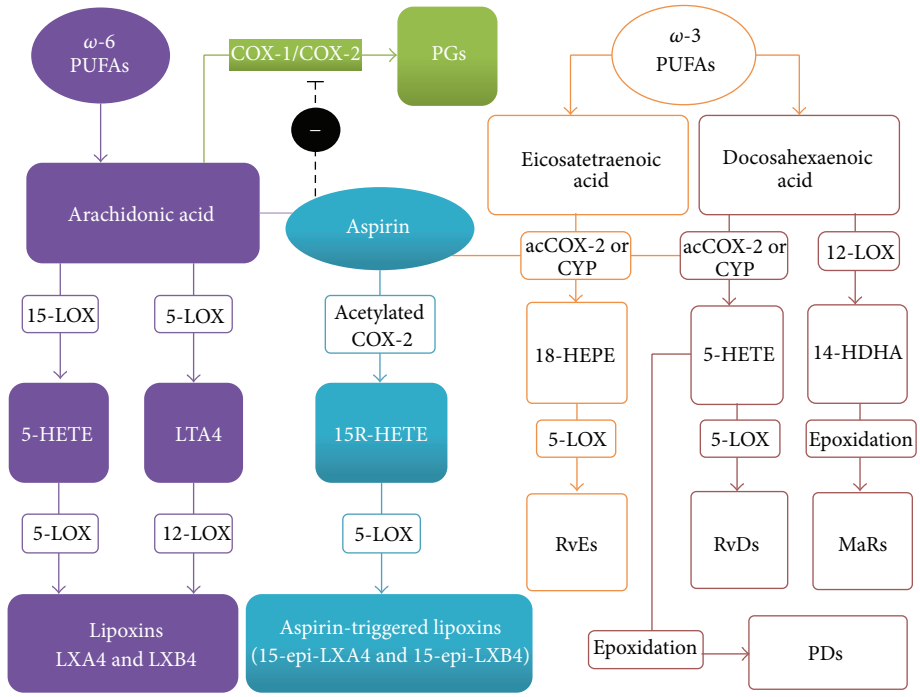

Figure 1: Schematic representation of the main biochemical pathways that mediate the production of proresolution lipid mediators. Arachidonic acid is derived from omega (ω)-6 and can be converted into lipoxins by lipoxygenases action. Omega (ω)-3 originates EPA-derived resolvins series E and DHA-derived resolvins series D, protectins, and maresins. COX: cycloxygenase; LOX: lipoxygenase; HETE: eicosatetraenoic acid; acCOX-2: acetylated cyclooxygenase-2; CYP: cytochrome P450; LXA4: lipoxin A4; RvEs: resolvins series E; RvD: resolvins series D; MaRs: maresins; PDs: protectins.

line with earlier observations that Aβ activates the NFκB signaling pathway in the mouse brain [34], ATLA4 treatment also reduced NFκB activation in brain astrocytes (but not in neurons or microglial cells) [34].

In summary, LXA4 and Aβ exert opposing effects at the ALX/FPR2 receptor, and whereas brain LXA4 production is reduced in AD, ALX/FPR2 receptors are overexpressed [29]. At first glance this scenario would strongly favor the strengthening action of Aβ on AD pathogenesis. However, paradoxically, the increased expression of ALX/FPR2 receptors in glial cells during AD should also render the diseased brain more responsive to LXA4, making the treatment with LXs a very interesting option for the AD therapy. Nonetheless, as LXA4 can also interact with additional receptors other than the ALX/FPR2 receptors, the impacts of LXA4 action on such molecular targets on its neuroprotective effects in AD remain to be better characterized. For example, considering that CB1 cannabinoids exert beneficial effects in animal models of AD [35], the fact that LXA4 is an allosteric signaling enhancer at CB1 cannabinoid receptors [19] might be relevant to its potential in AD treatment.

3. Stroke

Ischemic stroke is a major cause of morbidity and mortality throughout the world and its outcome depends on the extent of secondary brain damage to the penumbra caused by spreading inflammation [36]. Once a stroke occurs, permeability of the blood-brain barrier (BBB) promptly increases and activates a cascade of inflammatory responses which includes glial activation, neutrophil infiltration, increased expression of selectins and other intercellular adhesion molecules on BBB endothelial cells, as well as an infiltration of immune cells, leading to ischemic brain injury [37–39]. After stroke there is an excessive generation of reactive oxygen species (ROS) that aggravates neuronal death [40, 41]. The changes in BBB permeability seen shortly after the onset of transient or permanent focal ischemia in human patients and in animal stroke models are to a great extent the consequence of increased production of metalloproteinases (MMP), mainly of MMP-9 and MMP-2, by endothelial cells, microglia, and astrocytes [42–51]

As discussed previously, ALX/FPR2 receptors for LXA4 are present in neutrophils, monocytes, macrophages, neural stem cells, and resident cells in the CNS, which render them potential targets for LXA4 in the brain [52–55]. The initial inflammation seen shortly following injury gradually expands to affect a much larger area over several hours to days after a stroke [56, 57]. Brain ischemia rapidly triggers activation of resident glia alongside the recruitment of blood cells [58], and once neutrophils infiltrate the affected area they release phospholipases, proteases, and oxygenated free radicals [56]. Brain unsaturated fatty acids are especially

vulnerable to free radical-induced peroxidation. Not surprisingly, therefore, in animal models of stroke the injury can be ameliorated by blocking parts of the inflammatory cascade [59, 60] or limiting neutrophil infiltration at early stages [56, 58, 61].

Several studies have focused on the neuroprotective effects of central LXA4 treatment after stroke [38, 62–64]. Treatment of rats with LXA4 just after transient middle cerebral artery occlusion was found to reduce cerebral infarct volume, neutrophil infiltration, and neuronal apoptosis, and these effects were associated with a better neurological outcome [38]. Importantly, increases in glial cell activation and upregulation in the injured brain of the proinflammatory cytokines, IL-1β and TNF-α, which are so typical following stroke [65, 66], are also substantially reduced by LXA4 treatment [38, 62]. On the other hand, recovery from stroke has also been associated with upregulation of the anti-inflammatory cytokines, IL-10 and TGF-β1 [66, 67], and treatment with LXA4 or BML-111 (the stable synthetic LXA4 analogue 5(S),6(R)-LXA4 methyl ester) has been reported to increase the levels of such cytokines in stroke models involving both the peripheral and the central nervous systems [38, 68]. Such effects of LXA4 in stroke models have been associated with the suppression of NF-κB activation [38, 69, 70], an action which has been clearly evidenced in cultures of epithelial cells and human leukocytes [71, 72]. However, other studies have also implicated the activation of peroxisome proliferator-activated receptor (PPAR) Υ [73] and the upregulation of the antioxidant enzyme haeme oxygenase-1 (HO-1) and protein GSH [74] in the anti-inflammatory effects of LXA4 in stroke models.

The MMPs constitute another important target for the beneficial actions of LXA4 in stroke. In this regard, in rats subjected to transient middle cerebral artery occlusion, early postinjury treatment with the LXA4 analogue BML-111 promoted marked reductions in the expression and activity of MMP-9 and MMP-3, as well as an increase in expression of the endogenous MMP inhibitor TIMP-1 in the cortex [64]. This treatment also reduced brain edema, BBB disruption, and infarct size in the cortex, but not in the striatum, which suggests that it selectively attenuated spreading of inflammation throughout the cortex [64]. Moreover, BML-111 treatment dramatically reduced neutrophil infiltration into the brain and microglial cell activation [64]. Inhibition of glial cell activity might be particularly relevant to the anti-inflammatory activity of LXs as ATLA4 markedly reduces LPS-induced reactive oxygen species production in cultured microglial cells [75] and nitric oxide and PGE2 production by iNOS and COX-2 expression in cultured astrocytes [76].

To date only one study has attempted to use antagonists to characterize the receptors mediating the neuroprotective effects of LXA4 in stroke [74]. Of interest, that study showed that combined treatment of rats submitted to middle cerebral artery occlusion with the ALX/FPR2 receptor antagonist Boc-2 (butoxycarbonyl-Phe-Leu-Phe-Leu-Phe) only promoted partial blockade of LXA4-induced reduction in cerebral infarct size and improvement in neurological scores. Moreover, Boc-2 also failed to block LXA4-induced expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and its translocation to the nucleus, as well as that of HO-1 and synthesis of GSH. Indeed, an earlier study had shown that ALX4 activates the Nrf2 signaling pathway in mouse and human macrophages [77]. As this transcription factor coordinates the expression of genes regulated by antioxidant response elements, the Boc-2-resistant Nrf2-dependent effects of LXA4 described by Wu and collaborators [74], that is, increased expression of HO-1 (a redox-sensitive inducible enzyme) and synthesis of GSH (an antioxidant protein), constitute an important ALX/FPR2 receptor-independent mechanism to protect cells from oxidative damage following stroke.

Taken together, the studies reviewed in this section indicate that LXA4, ATLA4, and BML-111 all exert clear cut neuroprotective effects in stroke models. Thus, LXs might hold therapeutic value for the treatment of ischemic stroke. At least part of the neuroprotective effects of LXA4 appear to stem from activation of an Nrf2-GSH/OH-1 signaling pathway.

4. Traumatic Brain Injury

Traumatic brain injury (TBI) is defined as an alteration in brain function or evidence of brain pathology caused by an external force and is related with damage specifically to the brain [78]. An estimated 235,000 Americans are hospitalized annually for nonfatal TBI, and 1.1 million are treated in emergency departments, but, with 50,000 fatal cases every year, TBI is one of the leading causes of mortality among young people [79, 80]. The main causes of TBI include falls, vehicle accidents, assaults, and sports [81].

Surprisingly, the effects of LXA4 treatment in TBI have been largely unexplored. The only study published on this subject so far was carried out in mice subjected to a weight-drop model of TBI, in which the impact was directed to an exposed area of dura mater overlaying the cortex of the left cerebral hemisphere [82]. Injected into the ipsilateral lateral ventricle shortly after trauma, LXA4 was found to reduce BBB permeability, brain edema, and the extent of the lesion. Moreover, the magnitude of the increases in expression of mRNA and protein of the proinflammatory cytokines IL-1β, IL-6, and TNF-α was significantly smaller in extracts of lesioned cortex taken from LXA4-treated mice relative to TBI controls. The increases in phosphorylated ERK and JNK detected in injured cortex samples at 24 h after TBI were attenuated by LXA4 treatment. Interestingly, although TBI clearly enhanced the activation of cortical astrocytes (as estimated by GFAP immunofluorescence), without apparent change in activity of microglial cells, neither of these parameters were altered by LXA4 treatment. In addition, ALX/FPR2 receptor immunoreactivity seen within the layers of the injured cortex was greatly enhanced in comparison to the sham group and was mostly associated with astrocytes. Indeed, treatment with LXA4 actually increased ALX/FPR2 receptor expression selectively in astrocytes, even if it did not affect astrocyte activation by TBI.

Clearly, this pioneering study of Luo et al. [82] has already disclosed very encouraging actions of LXs in TBI and should stimulate much additional research on this particular topic.

5. Neuropathic Pain

The prevalence of chronic pain among the American and European population has been estimated to be around 30%, and about one-fifth of the people who report chronic pain are thought to suffer predominantly neuropathic pain (i.e., about 6% of the total population) [83]. Neuropathic pain is defined as pain resulting from injury to, or dysfunction of, the somatosensory system [84], but this terminology actually encompasses several types of neuropathic pain, most of which are poorly responsive to the drug treatments currently available [83].

Peripheral tissue injury or inflammation commonly triggers reversible changes in the sensory nervous system which enhance the sensitivity to nociceptive pain, a mechanism that protects and ensures proper healing of damaged tissue. By contrast, neuropathic pain is a frequently maladaptive condition resulting from direct injury to the nervous system itself. It is associated with persistent changes in sensitivity of pain pathways to perception of noxious stimuli, so that usually innocuous stimuli evoke pain (allodynia) and responses to noxious stimuli are exaggerated in amplitude (hyperalgesia) and/or duration (hyperpathy), alongside episodes of spontaneous pain [85].

The mechanisms underlying neuropathic pain development are numerous and diverse and frequently involve functional changes to both peripheral and central components of the pain pathways, even when the original injury is inflicted to primary sensory afferents in the periphery [79, 80, 85]. The peripheral sensitization to noxious stimulation is largely due to various alterations in expression and/or activity of ionic channels on nerve fibers, but we will briefly mention just a few of them. Neurotrophins and other mediators generated and released after peripheral nerve injury lower the activation threshold of heat- and acid-sensitive cationic TRPV1 channels and increase their expression not only in injured and uninjured C fibers but also in other primary afferents in which these channels are normally absent. Also, injury to primary sensory afferent fibers induces proliferation and redistribution of many subtypes of voltage-dependent sodium channels (such as Nav1.3, Nav1.7, and Nav1.8) and downregulates the expression and functioning of low voltage-activated and two-pore domain potassium channels. These changes in content and distribution of ion channels in primary afferent fibers are also important to generate ectopic discharges, which are thought to be responsible for neuropathic spontaneous pain. Peripheral nerve injury also induces neuroplastic changes in primary afferent neurons (such as phenotypic switches, collateral sprouting, and synaptic remodeling), augments glutamate release from their central terminals in the dorsal horn of the spinal cord, decreases its local uptake by glial cells, and stimulates spinal second-order nociceptive neurons to overexpress ionotropic NMDA receptors for glutamate. The ensuing potentiation of glutamatergic neurotransmission leads to a central (spinal) sensitization to pain, whereby the repetitive activation of primary afferent fibers causes a progressive increase in the frequency and magnitude of firing of dorsal horn second-order neurons, a phenomenon known as "windup." Neuropathic pain has also been associated with significant changes in the descending inhibitory and facilitatory controls exerted by supraspinal centers on the input of nociceptive information to the spinal dorsal horn.

Importantly, proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, are produced peripherally and centrally in response to nerve injury [86]. Therefore, peripheral and central neuroinflammation not only is implicated in the generation and maintenance of chronic inflammatory pain [79, 80] but also is likely to contribute to neuropathic pain [79, 80]. In fact, even if neuropathic and nonneuropathic pains are generally acknowledged to constitute distinct entities, many of the neurotransmitters, neuropeptides, cytokines, and enzymes implicated in both types of pain are the same [83]. In this regard, only a few studies have attempted so far to characterize the effects of LXs and ATLs in models of inflammatory and neuropathic pain.

The first study to assess the effects of LXA4 on pain found that intravenous or intrathecal injections of LXA4, LXB4, or an ATL analogue reduced inflammatory hind paw thermal hyperalgesia induced by carrageenan in rats [54]. The study also reported that spinal astrocytes express ALX/FPR2 receptors and respond to LXA4 with a diminished activation of extracellular signal-regulated kinase and c-Jun N-terminal kinase. Corroborating the view of a regulatory role for LXs in spinal inflammatory nociceptive processing, another study showed that intrathecal LXA4 administration also inhibits the mechanical allodynia and the increase in spinal TNF-α levels induced by carrageenan into the hind paw of rats [87].

On the other hand, LXs have also been found to be effective in models of neuropathic pain induced by peripheral nerve injury. In this regard, intrathecal LXA4 injection has been reported to reduce persistently the thermal hyperalgesia and mechanical allodynia which follow chronic unilateral compression of L4 and L5 DRGs in rats [79, 80]. These effects of LXA4 were associated with inhibition, in the compressed DRGs, of the NK-κB signaling pathway and mRNA levels for the proinflammatory cytokines IL-1β, IL-6, and TNF-α. In addition, repeated intrathecal ATLA4 administration to rats submitted to chronic constriction of sciatic nerve consistently reduced thermal hind paw hyperalgesia and significantly inhibited NALP1 inflammasome activation, caspase-1 cleavage, and IL-1β maturation in the spinal cord [79, 80]. Another recent study of the same group reported that the hind paw mechanical allodynia which occurs in the same model was reversed by single intrathecal injections of LXA4 or ATLA4 [79, 80]. The effects of both LXs were abrogated by administration of BOC-2, an ALX/FPR2 receptor antagonist, and most likely involved inhibition of the JAK2/STAT3 signaling pathway and attenuation in the upregulation of mRNA levels for IL-1β, IL-6, and TNF-α in the spinal cord. Importantly, the neuropathic procedure did not modify the content of ALX4 in neurons and astrocytes of the spinal dorsal horn, and the

degree of mechanical allodynia was unaffected by treatment with BOC-2 alone.

Direct lesions to the central nervous system, such as those inflicted by stroke in or traumatic injury to the brain or spinal cord, can also provoke a condition of neuropathic pain known as "central pain" in a significant proportion of patients [88]. The possible effects of LXs in controlling the nociceptive alterations and spontaneous pain associated with these types of injury remain to be estimated, but, from the studies reported in this section, the LXs may constitute a novel means to effectively target pain of both inflammatory and neuropathic pain.

6. Conclusions

Over the years, evidence that LXs exert potent neuroprotective and proresolution actions has been consolidated. The identification of their anti-inflammatory properties and effects altered the long-held initial belief that all AA-derived mediators are exclusively proinflammatory, and the evidence accumulated thus far indicates that LXs are powerful proresolving eicosanoids that can profoundly affect several aspects associated with AD, stroke, traumatic brain injury, and neuropathic pain. However, the potential impact of LXs and ATLs in pathological aspects of specific and important conditions, such as spinal cord injury, Parkinson's disease, and Huntington's disease, as well as in other neurodegenerative disorders of the central nervous system is still completely unknown. The studies summarized in the current overview underline the role of LXs in resolution and neuroprotection, but clearly a lot remains to be investigated in relation to the molecular targets of LXs and signaling pathways controlled by them. The development of new potent, selective, and long-acting pharmacological tools targeting different aspects of the LX system would greatly facilitate a better understanding of its importance in modulating diseases of the brain and spinal cord. The evidence available thus far qualifies the LXs as potent agonists for neuromodulation, neurological protection, and resolution of the diseased CNS and highlights the potential of treatments based on LXs in the management of neurodegenerative diseases affecting the brain and spinal cord.

Conflict of Interests

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

Authors' Contribution

Alessandra Cadete Martini and Stefânia Forner contributed equally to this work.

Acknowledgment

The authors are supported in Brazil by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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