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Leukotrienes Produced in Allergic Lung Inflammation Activate Alveolar Macrophages

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Key Words

Alveolar macrophages • Leukotrienes • Asthma • Killing • *Klebsiella pneumoniae*

Abstract

It has been well-documented that leukotrienes (LTs) are released in allergic lung inflammation and that they participate in the physiopathology of asthma. A role for LTs in innate immunity has recently emerged: Cys-LTs were shown to enhance FcγR-mediated phagocytosis by alveolar macrophages (AMs). Thus, using a rat model of asthma, we evaluated FcγR-mediated phagocytosis and killing of *Klebsiella pneumoniae* by AMs. The effect of treatment with a cys-LT antagonist (montelukast) on macrophage function was also investigated. Male Wistar rats were immunized twice with OVA/alumen intraperitoneally and challenged with OVA aerosol. After 24 h, the animals were killed, and the AMs were obtained by bronchoalveolar lavage. Macrophages were cultured with IgG-opsonized red blood cells (50:1) or IgG-opsonized *K. pneumoniae* (30:1), and phagocytosis or killing was evaluated. Leukotriene C_4 and nitric oxide were quantified by the EIA and Griess methods, respectively. The results showed that AMs from sensitized and challenged rats presented a markedly increased phagocytic capacity via FcγR (10X compared to controls) and enhanced killing of *K. pneumoniae* (4X higher than controls). The increased phagocytosis was inhibited 15X and killing 3X by treatment of the rats with montelukast, as compared to the non-treated group. cys-LT addition increased phagocytosis in control AMs but had no effect on macrophages from allergic lungs. Montelukast reduced nitric oxide (39%) and LTC_4 (73%). These results suggest that LTs produced during allergic lung inflammation potentiate the capacity of AMs to phagocytose and kill *K. pneumonia* via FcγR.

Introduction

Macrophages are the first line of defense and exhibit a variety of activities, including induction of inflammation, engulfment of microorganisms and dead cells, Ag presentation, and regulation of extracellular components. Alveolar macrophages (AMs) also produce and secrete inflammatory cytokines, chemokines and lipid mediators such as leukotrienes (LTs), and therefore, they play an important role in innate immunity [1, 2]. Leukotrienes are

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produced in response to a variety of infectious agents and enhance diverse leukocyte functions, including adherence, phagocytosis [3], secretion of reactive oxygen intermediates [4] and chemokine synthesis [5]. These effects of leukotrienes are so important that in animal models of infection, the pharmacological inhibition of leukotriene synthesis impairs the clearance of microorganisms and increases susceptibility to myriad different infections [3, 6-8].

Arachidonic acid (AA), a normal component of cell membrane phospholipids, is a substrate for prostaglandin endoperoxide (PGH) syntases-1 and -2, also known as cyclooxygenase (COX)-1 and -2, lipoxygenase (5-, 12-, or 15) (LO) or cytochrome p450 enzymes. Leukotrienes are produced in a multi-step enzyme pathway called the 5-lipoxygenase (5-LO) pathway, which is active in leukocytes such as neutrophils, eosinophils, mast cells and monocytes [9]. LTs exert their biological effects by activating specific receptors belonging to the superfamily of G protein-coupled receptors, including both LTB_4 and the cysteinyl LTs (cys-LTs) C_4, D_4, and E_4. Two receptors for LTB_4 have been identified: BLT1 and BLT2. BLT1 is a high-affinity receptor that is specific for LTB_4, which is expressed primarily in leukocytes and mediates chemotaxis; BLT2 is a pharmacologically distinct receptor that is ubiquitously expressed, displays a low affinity for LTB4 and binds to other eicosanoids. Receptors that are activated in response to Cys-LTs were cloned in 1999 and termed CysLT1 and CysLT2. CysLT1 recombinant receptor is activated by all of the native ligands with the following order of potency: LTD4 > LTC4 > LTE4. In contrast, the agonist rank order potency for the CysLT2 receptor is $LTD_4 = LTC_4$, with LTE_4 demonstrating less potency [10].

Leukotrienes are produced during allergic lung inflammation and are responsible for several inflammatory events. Their role in experimental models of allergic asthma has been well documented [11, 12]. Several studies have demonstrated that LTs are also relevant in human asthma, and the use of cys-LT antagonists has demonstrated beneficial effects. Cys-LTs are released from inflammatory cells that participate in the pathogenesis of allergic rhinitis [13], are elevated in patients with perennial allergic rhinitis [14], and are released following allergen exposure [15]. The CysLT1 receptor is expressed in nasal inflammatory cells and nasal mucosal glands [16], and CysLT administration reproduces the symptoms of allergic rhinitis. Moreover, CysLT1 receptor antagonists reduce the symptoms and signs of allergic rhinitis, reducing nasal allergic inflammation [13].

The phagocytosis of opsonized particles is triggered by the interaction of opsonins with specific receptors on the phagocyte surface. One of the most proeminent and well-studied phagocytic receptors is the Fcγ receptor (FcγR), which binds to the Fc portion of immunoglobulins (Ig) that coat the target [17]. LTB_4 and cys-LTs (LTD_4) have been shown to enhance FcγR-mediated phagocytosis by AMs [2].

Considering that cys-LTs are produced in asthmatic lungs and enhance the phagocytic and microbicidal effects of AMs, we investigated the phagocytic and microbicidal activities of macrophages from rat lungs undergoing allergic lung inflammation.

Materials and Methods

Animals

Eight to twelve-week-old Male Wistar rats (250-300 g) from our own colony were housed in a 22 ± 1°C environment at 60% humidity and were maintained on a 12-h light–dark cycle with food and water provided ad libitum. All procedures used in this study were approved and performed in accordance with guidelines established by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Biomedical Sciences Institute/USP–Ethical Committee for Animal Research (CEEA).

Immunization protocol

Rats were sensitized on days 0 and 7 by an intraperitoneal injection of a mixture containing 50 mg of ovalbumin and 1 mg of Al(OH)3 in saline (total volume of 0.7 ml). At day 14 after the first immunization, the animal were challenged by exposure to an aerosol of ovalbumin (grade III, Sigma) generated with an ultrasonic nebulizer (ICEL US-800, SP, Brazil) that delivers particles of 0.5–10 μm in diameter at approximately 0.75 ml/min for 20 min. The concentration of ovalbumin in the nebulizer was 2.5% (w/v).

The control group consisted of animals that were immunized as described and challenged with saline solution. The experimental groups of animals were treated 30 min before each challenge with effective doses of a cysteinyl-leukotriene receptor antagonist (montelukast, 5 mg/kg i.p.).

Bronchoalveolar lavage

Rats were killed with an overdose of anesthesia 24 h after exposure to aerosol challenge. A tracheal cannula was inserted via a midcervical incision, and the airways were lavaged five times with 10 ml of phosphate-buffered saline (PBS, pH 7.4 at 4°C).

Total and differential cell counts

The bronchoalveolar lavage fluid (BALF) was centrifuged at 170 g for 10 min at 4ºC, the supernatant was removed, and the cell pellet was resuspended in 0.5 ml of PBS. One volume of

a solution containing 0.5% crystal violet dissolved in 30% acetic acid was added to nine volumes of the cell suspension. The total number of cells was determined by counting using a hemocytometer. Differential cell counts were performed after cytocentrifugation and staining with hematoxylin and eosin (Hema 3).

Cell isolation and culture

Resident AMs from rats were obtained via *ex vivo* lung lavage as previously described [2] and resuspended in RPMI-1640 (Gibco-Invitrogen, Carlsbad, CA) to a final concentration of $2x10^5$ cells/mL. The cells were allowed to adhere to culture plates for 1 hour (37°C, 5% CO_2) followed by one wash with warm RPMI-1640. More than 99% of the adherent cells were identified as AMs according to a modified Wright-Giemsa stain. Cells were cultured overnight in RPMI-1640 supplemented with 2% fetal bovine serum (FBS) and then washed twice with warm medium to remove non-adherent cells.

Phagocytosis of red blood cells opsonized with IgG and evaluation of the phagocytic index

Red blood cells (Bioboavista Laboratories, Brazil) were opsonized with a subagglutinating concentration of IgG rabbit anti-sheep erythrocyte antibody (IgG-RBCs) (Cappel Organon Teknika, Durham, NC) as previously described [18].

The phagocytosis of lamb red blood cells by rat AMs was evaluated according a method previously described by Aronoff et al. [19]. Briefly, 2 x 10^5 cells were added to each well of a 24-well culture plate and allowed to adhere for 1 hour (37°C, 5% CO_2). The cell monolayer was washed to remove non-adherent cells and then cultured for 18 hours in RPMI containing 2% fetal bovine serum (HyClone, Logan, UT-USA), according to Serezani et al. [2].

The alveolar macrophages were washed with PBS at 37°C and incubated with or without LTC_4 (100 nM) (Biomol Inc., Palo Alto, CA-USA) for 10 minutes. Following the incubation, the supernatant was removed, and opsonized blood cells or not opsonized (internalization control) were added to the AMs at a concentration ratio of 50:1. The samples were incubated for 90 min at 37°C and 5% CO_2 in PBS. After phagocytosis, the cells were washed three times with PBS and stained with hematoxylin and eosin. The phagocytic index (PI), which was derived by multiplying the percentage of positive macrophages (AMs containing at least one ingested target) by the mean number of phagocytosed targets per positive macrophage, was determined by optical microscopy (x1000).

Bacterial killing assay

Klebsiella pneumoniae 43816, serotype 2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Bacterial stocks were thawed, inoculated into broth, and grown to log phase. The internalization and intracellular killing of *K. pneumoniae* was monitored using a modification of the assay described by Bidani et al. [20]. Briefly, $2x10^5$ AMs were suspended in RPMI and infected with *K. pneumoniae* at a concentration ratio of 30:1 bacteria per cell at 37ºC for 1 h. The cell suspensions were centrifuged (300 xg, 10 min, 4ºC), and then the supernatants were collected, and the cells were washed twice in PBS and lysed. Quantitative bacterial cultures were performed for the supernatants and cell lysates to determine the colony-forming units (CFUs) in the extracellular and intracellular cell environments. CFUs were determined by serial dilution on agar plates. *K. pneumoniae* was grown in BBL brain heart infusion broth and nutrient agar (DIFCO; Becton Dickinson) and incubated at 37ºC for 18–24 h.

Measurement of nitric oxide

To evaluate nitric oxide (NO) production, the nitrite concentrations in the supernatants of the AM cultures were measured using the standard Griess reaction [21]. Briefly, 50 µl of supernatant culture medium was added to 50 µl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 2.5% (v/v) phosphoric acid and 0.1% (w/v) naphthylenediamine-HCl] and incubated at room temperature for 10 min. The absorbance at 540 nm was then measured in a microplate reader Multiskan^{EX}, and the nitrite concentration was calculated based on a standard curve of sodium nitrite. All assays were performed in triplicate.

Extraction and quantification of leukotrienes

The octadecylsilyl silica column (Sep-Pak C18 column) was pre-washed with 10 ml of ethanol and 10 ml of water, and the supernatants from homogenized and acidified lung tissues (pH 3.4–3.6 with HCl 1 N) were allowed to pass slowly through the column. The column was then washed with 10 ml water and 1 ml ethanol (35%), and the eicosanoids were eluted from the column with 2 ml absolute ethanol. The samples were dried under a stream of nitrogen. LTC_4 and LTD_4 concentrations were determined using EIA kits (Cayman Chemical Co., MI, USA) according to the method reported by Pradelles et al. [22]. Briefly, dilutions of the supernatants were incubated with the conjugated eicosanoid–acetylcholinesterase complex and with specific antiserum in 96-well plates precoated with anti-rabbit immunoglobulin G antibodies. After an overnight incubation at 4ºC, the plates were washed, and the enzyme substrate (Ellman's reagent) was added for 60–120 min at 25ºC. The optical density of the samples was determined at 412 nm using a microplate reader, and the concentration of eicosanoids was calculated based on a standard curve.

Statistical analysis

Data are expressed as the means ± SEM. Statistical analyses were performed using the GraphPad software (San Diego, CA, USA) and compared by analysis of variance (ANOVA) followed by the Bonferroni test or the unpaired Student *t* test where appropriate. A p value less than 0.05 was considered statistically significant. All of the experiments were performed at least three independent times.

Results

Effect of montelukast on lung cell infiltration

Rats immunized with ovalbumin were submitted to ovalbumin aerosol challenge, and bronchoalveolar lavage was performed 24 h after the aerosol challenge. The

Fig. 1. Effect of montelukast on bronchoalveolar lavage cells. Rats were immunized with OVA and challenged with OVA aerosol. Montelukast was given i.p. 30 min. before aerosol challenge. Bronchoalveolar lavage was performed 24 h after challenge. Eosinophils and neutrophils were counted in a cytocentrifuge preparation of bronchoalveolar lavage cells stained with hematoxylin and eosin. The results represent the mean ± S.E.M. of 6 animals per group. * P< 0.01 compared with the control group, and # P< 0.05 compared with the non-treated group.

control group consisted of immunized and saline challenged rats.

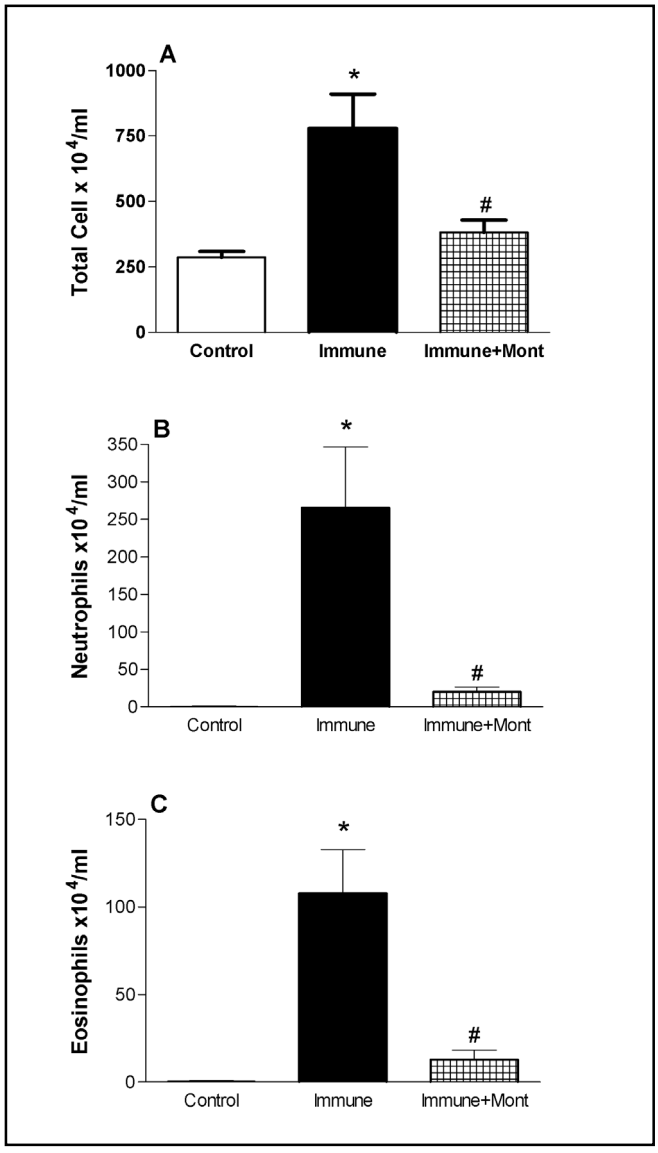
Whereas the numbers of eosinophils and neutrophils detected in the BALF of the control group were very low (less than 1 cell/ml), significant increases in total cells (287.5 ± 21.8 to 778 ± 131.2 cells x10^4/ml), neutrophils (0.5 ± 0.2 to 265.2 ± 81.5 cells x10^4/ml) and eosinophils (0.5 ± 0.2 to 107.8 ± 25 cells x10^4/ml) were observed in the immunized and antigen-challenged rats (Fig. 1A, B and C). The groups of immunized rats received intraperitoneal injections of selected effective doses of antagonists of cysteinyl-leukotrienes (Montelukast, 5 mg/kg) 30 min before aerosol antigen challenges. Figure 1A, B and C shows that all treatments significantly decreased the numbers of total cells (50%), neutrophils (92%) and eosinophils (88%) in the bronchoalveolar lavage fluid of rats following treatment with the cysteinyl-leukotriene receptor antagonist.

Effect of montelukast on leukotriene production in lung tissue

The lung tissues were homogenized and centrifuged, and the LTB_4 and LTC_4/D_4 concentrations were determined using EIA kits. The animals that were immunized and sensitized demonstrated increased levels of LTB_4 (2665 ± 160.2 to 28060 ± 808 pg/ml) and LTC_4/D_4 (1152 ± 159 to 8645 ± 771.8 pg/ml) as compared to the control groups. The rats treated with montelukast displayed significantly reduced levels of LTC_4/D_4 (8645 ± 771.8 to 2331 ± 912.4), as shown in Fig. 2; however, pre-treatment with montelukast did not significantly affect LTB_4 production (data not shown).

Effect of montelukast on alveolar macrophage phagocytosis

Macrophages from sensitized rats showed increased phagocytosis eight hours after incubation with lamb red blood cells, as compared to the control group (255 ± 45.5 to 826.5 ± 91.6), and this increase



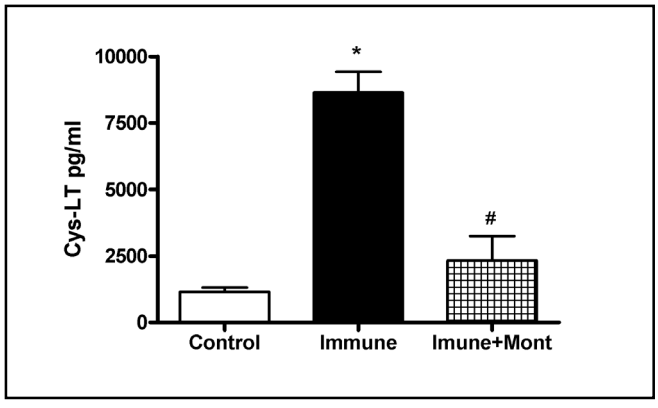


Fig. 2. Cys-leukotriene production in lung tissue and effect of Montelukast. Leukotriene production was measured using the EIA kit. The results represent the mean ± S.E.M. of 6 animals per group. * P< 0.01 compared with the control group, and # P< 0.05 compared with the immunized group.

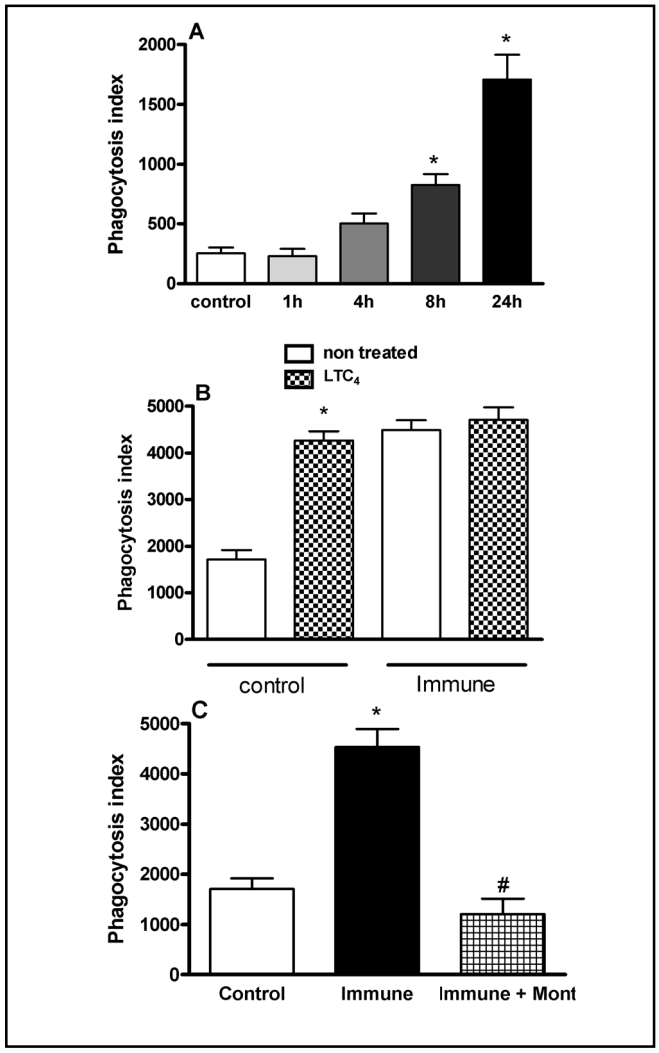


Fig. 3. Phagocytosis assay of red blood cells opsonized with IgG and evaluation of the phagocytic index. Resident macrophages from immunized male Wistar rats were obtained by ex vivo bronchoalveolar lavage. (A) shows that AMs from sensitized rats demonstrated a significant increase in phagocytosis via FcγR that persisted up to 24 h. (B) shows that the addition of LTs increased the rate of phagocytosis, and C shows that montelukast reduced the rate of AM phagocytosis in the immunized group. The results represent the mean ± S.E.M. of 6 animals per group. * P< 0.01 compared with the control group, and # P< 0.05 compared with the immunized group.

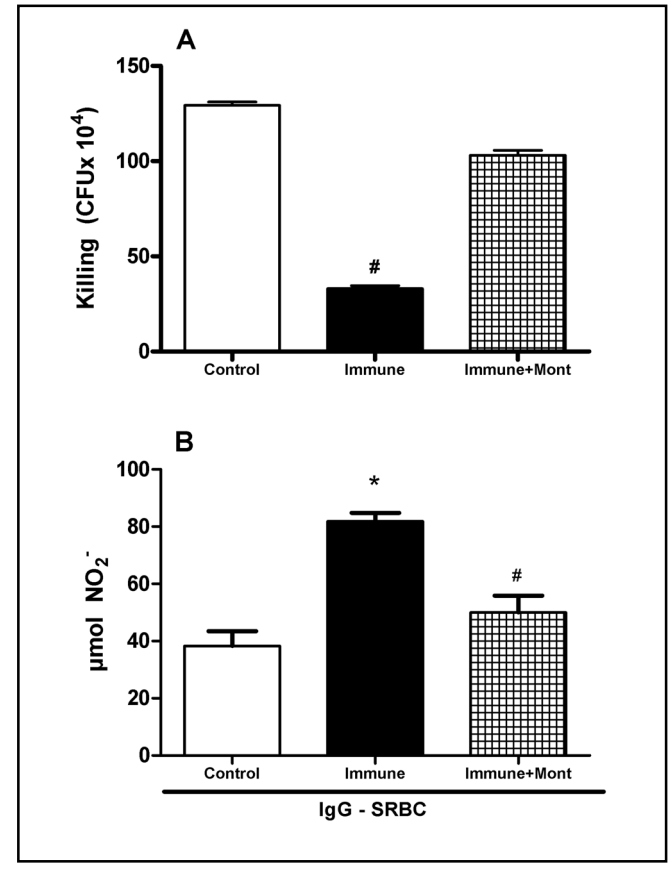


Fig. 4. (A) - Killing of *K. pneumoniae* by alveolar macrophages and effect of montelukast. AMs were infected with *K. pneumoniae in vitro.* After 3 hours, the macrophages were removed by scraping, and aliquots were cultured to determine the number of viable organisms by quantitative culture. (B) shows the production of nitric oxide in culture supernatants and the effect of montelukast. Nitric oxide production was measured using the Griess colorimetric reaction following the addition of sheep red blood cells (SRBCs) to AMs. The results represent the mean ± S.E.M. of 6 animals per group. * P< 0.05 compared with the control group, and # P< 0.05 compared with the immunized group.

persisted up to 24 h (255 ± 45.5 to 1709 ± 207.3) (Fig. 3A). The addition of cys-LTs resulted in an increased rate of AM phagocytosis (149%) in the control group. Immunized and challenged rats showed similar phagocytosis indices when compared to control animals stimulated with LTC_4; however, the phagocytic capacity of this experimental group did not increase in response to stimulation with LTC_4 (Fig. 3 B). Figure 3 C shows that treatment with the cys-LTS antagonist reversed the increased phagocytic index of alveolar macrophages from immunized and challenged rats.

Effect of montelukast on macrophage killing and nitric oxide generation

An increased rate of killing of internalized bacteria was observed in AMs from sensitized rats (128 ± 2 to 33

± 1.5 CFU x 10^4); however, AMs collected from sensitized rats treated with a cys-LTS antagonist prior to antigen challenge showed a reduced killing capacity (33 ± 1.5 to 103 ± 2.6 CFU x 10^4) (Fig. 4A).

The culture supernatant of macrophages from rats sensitized contained higher levels of nitric oxide compared to the control group (38.3 ± 5.2 to 81.9 ± 3 μmol NO_2^-). However, the pre-treatment of immunized rats with montelukast significantly reduced nitric oxide production in the culture supernatants of AMs (81.9 ± 3 to 50 ± 5.8 μmol NO_2^-) (Fig. 4B).

Discussion

The present study established the importance of LTs in the phagocytic and microbicidal activities of macrophages from rat lungs undergoing allergic lung inflammation. Moreover, we demonstrated the relevance of endogenous and exogenous LTs in the in vitro activities of alveolar macrophages and in the production of NO.

It has been well-established that LTB_4 and Cys-LTs are potent chemotactic factors involved in the recruitment of cells at the inflammation site [23-25]. Cuzzocrea et al. [26] showed decrease in neutrophil infiltration due to the absence of 5-LO, and aerosolized cys-LTs elicit the recruitment of eosinophils to the lung in guinea pigs, which is blocked by Cys-LT1 antagonists [27, 28]. Consistent with these data, our results demonstrated that in rats treated with montelukast, there was a direct relationship between leukocyte infiltration and Cys-LT production in the lung tissue. Moreover, in rats pretreated with montelukast, we also observed a reduced infiltration of polymorphonuclear cells in the lung tissue, as evaluated by histological examination (data not shown). The reduced levels of LTC_4 observed in the lung tissue of immunized and challenged rats pretreated with the cys-LT antagonist corroborate the results of our previous studies [11] and can be explained by the reduced infiltration of inflammatory cells in lung tissue following montelukast treatment. Polymorphonuclear cells produce leukotrienes, and the reduced cell infiltration after treatment with montelukast could explain the decrease in leukotrienes levels observed in the lung tissue.

It has been demonstrated in different infection models that LTs increase the effector functions of phagocyte, including phagocytosis, microbicidal activity, the generation of reactive oxygen and nitrogen species, and secretion of proinflammatory cytokines [1]. It has also been established that LTs increase the phagocytosis of IgG- and complement-opsonized targets as well as microbicidal activity and amplify a number of relevant signal transduction events [2, 29].

We demonstrated that macrophages from rats sensitized and challenged with ovalbumin aerosol had an increased capacity to phagocytosis IgG- and complement-opsonized targets and to kill *K. pneumoniae*, and treatment of the rats with montelukast abolished these events. The main finding of the present study is that treatment with montelukast decreased the production of LTC_4 by lung tissue. We propose that this reduction could be related to the functional impairment in *in vitro* phagocytosis and microbicidal activity observed in macrophages from immunized and challenged rats.

The ligation of Fc receptors not only results in phagocytosis but also the synthesis and release of O_2^- and H_2O_2, which are powerful oxidants that either alone or in concert with leukocyte enzymes efficiently kill pathogens [30]. In fact, the superoxide anion (O_2^-) and nitric oxide have been identified as critical for the control of leishmania infections [31].

Although the studies cited above have provided a vast amount of information about the role of NO in bacterial pathogenesis, caution is needed in extrapolating these findings to human cells, because iNOS expression and the production of NO by AMs differ dramatically among species [32]. The ability of AMs to produce NO and to kill bacteria has been alluded to in a number of clinical studies [33-36]; however, the majority of NO bacterial pathogenesis studies have relied on information obtained using rodent models, reinforcing the importance of animal models [37-39]. In the present study, AMs from immunized and challenged rats demonstrated a significant increase in production NO in response to a stimulus, and treatment with montelukast abolished this event. The addition of LTC_4 on AMs culture stimulated a significant increase in NO production and *K. pneumoniae* killing. In fact, the relationship between nitric oxide (NO) and cysteinyl LTs has been demonstrated in previous studies. Yang et al. [40] have shown that sodium nitroprusside, a nitric oxide donor, down-regulates the mRNA expression of LTC_4 synthesis enzymes in hepatic ischemia/reperfusion injury in rats via the NF-kappaB signaling pathway. Our results are consistent with those of Ko and Cho [41], who observed a reduction in the level of LTC_4 after administration of L-NAME (an inhibitor of nitric oxide synthesis). However, the regulatory mechanism of these mediators remains unknown.

Taken together, these results suggest that LTs produced during allergic lung inflammation can potentiate the capacity of alveolar macrophages to phagocytose and eliminate *K. pneumoniae* via FcγR. These results raise the possibility that asthmatics might be more resistant to infections caused by airway pathogens, and thus, treatment with LTs antagonists could abolish this protective effect of allergy.

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