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RESEARCH ARTICLE

Cyclooxygenase-derived mediators regulate the immunological control of *Strongyloides venezuelensis* infection

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Abstract

The aim of this study was to define the immunoregulatory role of prostaglandins in a mouse model of *Strongyloides venezuelensis* infection. *Strongyloides venezuelensis* induced an increase of eosinophils and mononuclear cells in the blood, peritoneal cavity fluid, and bronchoalveolar lavage fluid. Treatment with the dual cyclooxygenase (COX-1/-2) inhibitors indomethacin and ibuprofen, and the COX-2-selective inhibitor celecoxib partially blocked these cellular responses and was associated with enhanced numbers of infective larvae in the lung and adult worms in the duodenum. However, the drugs did not interfere with worm fertility. Cyclooxygenase inhibitors also inhibited the production of the T-helper type 2 (Th2) mediators IL-5, IgG1, and IgE, while indomethacin alone also inhibited IL-4, IL-10, and IgG2a. Cyclooxygenase inhibitors tended to enhance the Th1 mediators IL-12 and IFN-γ. This shift away from Th2 immunity in cyclooxygenase inhibitor-treated mice correlated with reduced prostaglandin E_2 (PGE_2) production in infected duodenal tissue. As PGE_2 is a well-characterized driver of Th2 immunity, we speculate that reduced production of this lipid might be involved in the shift toward a Th1 phenotype, favoring parasitism by *S. venezuelensis*. These findings provide new evidence that cyclooxygenase-derived lipids play a role in regulating host defenses against *Strongyloides*, and support the exploration of eicosanoid signaling for identifying novel preventive and therapeutic modalities against these infections.

Introduction

Strongyloides stercoralis is a widespread infection that afflicts 30–100 million people in 70 countries, mainly in tropical and subtropical regions of the world (Genta, 1989; Siddiqui & Berk, 2001). This parasite can replicate within the human host, allowing infection to perpetuate and persist for several decades (Grove, 1996). Infection usually occurs following cutaneous penetration by infective larvae [third-stage larvae (L3)], but parasites may also transform into an infective stage inside the host gastrointestinal tract, termed autoinfection. Uncontrolled autoinfection of *S. stercoralis* is more likely to occur in immunosuppressed patients, leading to disseminated hyperinfection that is fatal in nearly 90% of cases (Siddiqui & Berk, 2001).

In human hosts and in murine models, the immune response to *Strongyloides* spp. is characterized by a T-helper type 2 (Th2) phenotype, with intraepithelial and tissue increases of eosinophils (Machado *et al.*, 2005; Mir *et al.*, 2006), as well as intestinal mastocytosis (Koyasu *et al.*, 2005; Sasaki *et al.*, 2005), production of Th2-type cytokines such as IL-3, IL-4, IL-5, IL-13 (Kobayashi *et al.*, 1998; Mir *et al.*, 2006), chemokines such as eotaxin (Mir *et al.*, 2006), and increased synthesis of immunoglobulin A (IgA), IgE, IgG, and IgM antibodies (Rossi *et al.*, 1993; Machado *et al.*, 2005).

Little is known about the role of lipid mediators in regulating the immune response to *Strongyloides* infections. Recently, Machado *et al.* (2005) demonstrated an increase in arachidonic acid (AA) metabolites in the lungs and intestines

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of *Strongyloides venezuelensis*-infected mice, including both the 5-lipoxygenase (5-LO)- and the cyclooxygenase -derived leukotrienes and prostaglandins, respectively. A protective role for the leukotrienes was observed in that study, with an increase in parasite burden and a shift away from appropriate Th2-type immune responses in mice treated with a 5-LO inhibitor or genetically deficient for 5-LO (Machado *et al.*, 2005). Although lung prostaglandin E_2 (PGE_2) levels were found to be elevated, prostaglandins were not investigated for their immunomodulatory role in that study.

The prostaglandins are cyclooxygenase-derived AA metabolites involved in diverse pathological, inflammatory, and physiological mechanisms. Key prostaglandin compounds include PGD_2, PGE_2, PGI_2 (prostacyclin), $PGF_{2\alpha}$, and thromboxane A_2 (TXA_2). Two isoforms of cyclooxygenase, COX-1 and COX-2, catalyze a two-step reaction that converts AA into the unstable intermediate PGH_2, which is further isomerized by specific synthase enzymes to the terminal prostaglandins (Vane *et al.*, 1998). In general, COX-1 is constitutively expressed in most nucleated cells, while COX-2 is not detected in uninflammed tissues, except the lung, brain, and kidneys. COX-2 expression is considerably enhanced in the setting of inflammation (Wallace, 2001; Miller, 2006).

An immunoregulatory role for prostaglandins in parasitic infections has been established for a diverse array of pathogens, including *Entamoeba histolytica* (Stenson *et al.*, 2001), *Leishmania amazonensis* (Lonardoni *et al.*, 2000), *Angiostrongylus costaricensis* (Bandeira-Melo *et al.*, 2000), *Nippostrongylus brasiliensis* (Dineen & Kelly, 1976), and *Schistosoma mansoni* (Ramaswamy *et al.*, 2000; Zou *et al.*, 2007). These parasites induced the production of prostaglandins within the infected host that modulated the immunological response to and resolution of parasitic infection. Moreover, parasite-derived prostaglandins have been shown to regulate the immune defenses of the host. For example, the production of PGD_2 by the helminthic parasite *S. mansoni* inhibited the migration of epidermal Langerhans cells to draining lymph nodes (Angeli *et al.*, 2001), and pivotal roles for parasite PGD_2 synthesis and host D prostanoid receptor 1 have been established in schistosome immune evasion (Hervé *et al.*, 2003). Nevertheless, nothing has been reported on the role of the prostaglandins in strongyloidiasis.

In this study, we investigated the effects of cyclooxygenase-derived lipid mediators on *S. venezuelensis* infection in mice. Our results reveal that prostaglandins counterregulate effective immune responses against *S. venezuelensis* in mice.

Materials and methods

Animals

Male Swiss mice weighing 16–20 g (and aged 21–30 days) and male *Rattus norvegicus* (Wistar) rats weighing 120–180 g were obtained from the animal facilities of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo (USP), Brazil. All experiments were approved by and conducted in accordance with guidelines established by the CEUA Animal Care Committee (protocol no. 02.1.1408.53.8). All animals were maintained under standard laboratory conditions.

Parasites

The strain L-2 of *S. venezuelensis* was originally isolated from the wild rodents *Bolomys lasiurus* in April, 1986. This strain was maintained in Wistar rats, routinely infected in the Laboratory of Inflammation and Immunology of Parasitosis at the Faculdade de Ciências Farmacêuticas de Ribeirão Preto – USP, São Paulo, Brazil.

Infection of mice with *S. venezuelensis* and treatment with cyclooxygenase inhibitors

Infective L3 of *S. venezuelensis* were obtained from charcoal cultures of infected rat feces. The cultures were stored at 28 °C for 72 h, and the infective larvae were collected and concentrated using a Baermann apparatus. The recovered larvae were then washed several times in phosphate-buffered saline (PBS) and counted. The number was subsequently adjusted to 16 000 L3 per milliliter of PBS for infection. Swiss mice were individually inoculated via a subcutaneous abdominal injection with 100 μL of PBS containing 1500 *S. venezuelensis* L3. The Swiss mice were divided into five groups. Mice in one group were treated orally by a gavage with 0.5 mL of a 10 mg kg^{-1} solution of the COX-1/-2 inhibitor indomethacin (Sigma-Aldrich, St. Louis, MO) solution. Mice in a second group received 15 mg kg^{-1} in 0.5 mL of the COX-1/-2 inhibitor ibuprofen (Sigma-Aldrich). A third group was treated with 15 mg kg^{-1} in 0.5 mL of the COX-2-selective inhibitor celecoxib (Sigma-Aldrich). The doses of these drugs were defined according to previous publications (Niederberger *et al.*, 2001; Shishodia & Aggarwal, 2004). The first and last treatment doses of medication were administered 1 h before infection and then daily until day 21 (final evaluation day). In a fourth group, the mice were treated orally by a gavage with 0.5 mL of water. In a fifth group, the mice were used as controls (uninfected and untreated). The last treatment was given 1 h before sacrifice. The experiment was repeated twice and the data are expressed as mean ± SEM from two representative experiments ($n = 6$–8 per day) for infected mice and ($n = 5$–6 per day) for uninfected mice.

Collection of blood, serum, bronchoalveolar lavage fluid (BALF), and peritoneal cavity fluid (PCF)

On postinfection days 5, 7, 14, and 21, mice were anesthetized via a subcutaneous injection of 30 mg kg^{-1} of

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tribromoethanol (Acros Organics, Fairlawn, NJ), and blood samples were collected by cardiac puncture. Subsequently, mice were sacrificed with an overdose of tribromoethanol. The chest cavity of each animal was carefully opened, after which the trachea was exposed and catheterized. The catheter was tied in place, and sterile PBS/sodium citrate (0.5%) was infused in three 1-mL aliquots. The BALF was collected and placed on ice. The PCF was obtained by injecting 3 mL of PBS into the peritoneal cavity. Total cell counts in blood, BALF, and PCF were immediately performed in a Neubauer chamber. Differential counts were obtained using Rosenfeld-stained cytospin preparations or smears (Machado *et al.*, 2005). Blood was then centrifuged, and the serum was stored at $-70\,^{\circ}$C.

Filariform larvae, eggs, and adult worm counts

On postinfection days 3, 5, 7, 14, and 21, groups of mice were sacrificed and the lung, spleen, kidney, heart, liver, and brain were harvested. These organs were dissected so that migrating larvae could be collected and counted (Yoshida *et al.*, 1999). On postinfection days 5, 7, 14, and 21, mice were placed individually on a clean, moist absorbent paper and allowed to defecate. Eggs per gram of feces were counted using the Cornell–McMaster quantitative method (Machado *et al.*, 2003). The parasitological exam was performed twice, and the mean of the two results was recorded. The mice were then sacrificed with tribromoethanol. To count the adult parasites, 10 cm duodenal sections were subsequently removed, placed on Petri dishes containing saline, longitudinally sectioned, and incubated for 2 h at 37 °C. The adult worms from the intestine and filariform larvae from the lungs, spleen, kidney, heart, liver, and brain were counted under light microscopy at a magnification of $\times 100$.

Histology

Duodena (10-cm sections) and lung fragments were removed on postinfection days 3, 5, 7, and 14. The tissue samples were then fixed in 10% formalin and embedded in paraffin blocks. To count inflammatory cells and determine worm burdens, 5-μm sections were stained with H&E and analyzed in a blinded fashion.

Alkaline parasite extracts

Alkaline extracts were prepared as described previously in the literature (Machado *et al.*, 2003). In brief, 1 mL of 0.15 M NaOH was added to ~171 000 *S. venezuelensis* larvae, which were maintained under gentle agitation for 6 h at 4 °C. Subsequently, 0.3 M HCl was added until a pH of 7.0 was reached. This preparation was then centrifuged at 12 400 ***g*** for 30 min at 4 °C. The protein concentration of the supernatant was 1.96 mg mL^{-1}, as detected using the Lowry method (Lowry *et al.*, 1951). The antigenic extract was used for determination of antibodies in the sera.

Measurement of antibodies in sera

Specific IgG1, IgG2a, and IgE were determined in mice sera using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). The plates were coated with *S. venezuelensis* alkaline extract at a concentration of 20 μg mL^{-1} (50 μL per well), and the assay was carried out according to a procedure described in the literature (Lowry *et al.*, 1951; Machado *et al.*, 2005). The results are reported as the mean absorbance of samples per group (± SEM).

Measurement of PGE_2 and cytokines

For determination of PGE_2 and cytokine levels, the lungs and small intestine were removed on postinfection days 5, 7, 14, and 21. Tissue samples were homogenized (Ultra-Turrax T8; IKA-Werke, Staufen, Germany) in 1.5 mL (for lungs) or 2 mL (for duodena) of medium with protease inhibitors [aprotinin (5 μg mL^{-1}), leupeptine (100 mM), benzamidine hydrochloride (0.1 mM), pepstatin (10 μg mL^{-1}), PMSF (1 mM), EDTA (1 mM), Amresco, OH] (both lungs and intestine received proteases inhibitors) centrifuged at 1500 ***g***, filtered, and stored at $-70\,^{\circ}$C until assay. PGE_2 was quantified using a commercial ELISA kit obtained from Amersham Biosciences. Commercially available ELISA antibodies were used to measure IL-4, IL-5, IL-10, IL-12, and IFN-γ according to the manufacturer's instructions (BD Pharmingen). Sensitivities were > 10 pg mL^{-1}.

Statistical analysis

Each experiment was performed twice. The results of the experiments are expressed as mean ± SEM. Statistical variations were analyzed using ANOVA, followed by the Bonferroni test. Student's *t*-test was used in the analysis of parasite and egg numbers. The level of statistical significance was set at $P < 0.05$.

Results

Duodenal PGE_2 is increased in *S. venezuelensis*-infected mice and is subject to pharmacological suppression

We previously reported increased lung PGE_2 levels following *S. venezuelensis* infection (Machado *et al.*, 2005). To confirm enhanced PGE_2 release during *S. venezuelensis* infection, duodena of Swiss mice, treated or not with indomethacin, ibuprofen, or celecoxib, were harvested daily from postinfection days 5 to 21. Our results demonstrate that, on

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postinfection days 5, 7, 14, and 21, respectively, PGE_2 production in the duodena of mice infected with *S. venezuelensis* was significantly increased when compared with uninfected mice. Cyclooxygenase inhibitors significantly decreased the synthesis of PGE_2 in duodenal tissue when compared with infected mice. The mean inhibition of PGE_2 levels in infected mice treated with indomethacin, ibuprofen, or celecoxib was 26%, 12%, and 13%, respectively (Fig. 1).

Inhibition of prostaglandin production is associated with increased parasite burdens

Table 1 shows the number of infective larvae recovered from the lungs and the number of adult worms visualized in the histological cuts of the duodena of mice infected with *S. venezuelensis* treated or not with cyclooxygenase inhibitors. Larvae in the lungs of the mice infected and treated were enumerated on days 1, 3, 5, 7, 14 and 21 postinfection. The recovery of these parasite forms in the lungs was significantly higher in the three groups of treated mice when compared with untreated, infected mice on days 3, 5, and 7 postinfection.

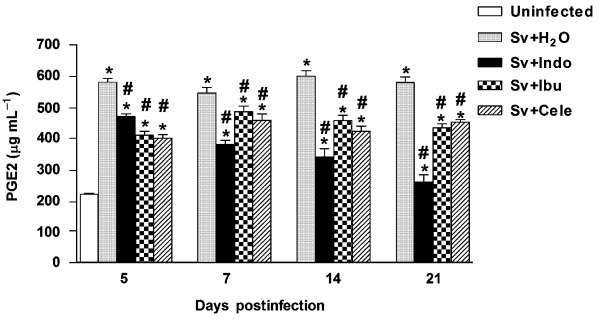

Fig. 1. Effect of cyclooxygenase inhibitors on PGE_2 synthesis in the duodena. PGE_2 levels were measured by ELISA. Swiss mice were infected subcutaneously with *S. venezuelensis* (Sv) larvae, and treated daily with water, or indomethacin (Indo), ibuprofen (Ibu), or celecoxib (Cele). Duodena were removed on postinfection days 5, 7, 14, and 21. A group of uninfected mice was used as a control. Data are expressed as mean ± SEM of two independent experiments ($n = 6$–8 mice per day) and uninfected mice ($n = 5$–6 per day). $^{*\#}P < 0.05$. *Uninfected control mice vs. Sv+H_2O, or Sv+Indo, or Sv+Ibu, or Sv+Cele; # Sv+H_2O vs. Sv+Indo, or Sv+Ibu, or Sv+Cele.

In the cyclooxygenase inhibitor-treated mice, parasites were observed in the small intestine on day 3 postinfection. The same trend (although not statistically significant) was seen on days 5 and 7 postinfection. Compared with infected mice not treated with cyclooxygenase inhibitors, the respective percentages of parasites recovered on days 5 and 7 were 32% and 10% higher for indomethacin, 25% and 21% higher for ibuprofen, and 24% and 20% greater for celecoxib (Fig. 2a). Cyclooxygenase inhibitors increased the retention of adult worms in the intestine of infected hosts. In untreated, infected mice, adult parasites were not recovered from the intestine on day 14. However, in the cyclooxygenase inhibitor-treated animals, small numbers of female adult worms were consistently recovered on this later date postinfection. These results were confirmed in the histological cuts of the duodena (Fig. 3). We examined other organs such as the liver, spleen, heart, and brain during all kinetics of the infection for detection of eggs, larvae, and parasitic females, but in these organs, eggs and larvae were not observed. These results suggest that prostaglandins are important in the protection of the infected host during strongyloidiasis.

There was a statistically significant tendency toward an increase in the number of *S. venezuelensis* eggs per gram of feces, observed on days 5 and 7 postinfection in the cyclooxygenase inhibitor-treated mice. The percentages

Table 1. Treatment with cyclooxygenase inhibitors increase percentage of permanence of infective larvae in the lungs and adult worms in the duodena of the mice infected with *Strongyloides venezuelensis* in histopathological study

Days	Sv+H_2O		Sv+indomethacin		Sv+ibuprofen		Sv+celecoxib	
postinfection	Lungs (larvae)	Duodena (adult)	Lungs (larvae)	Duodenal (adult)	Lungs (larvae)	Duodena (adult)	Lungs (larvae)	Duodena (adult)
1	77 ± 2	0 ± 0	69 ± 4	0 ± 0	92 ± 5	0 ± 0	61 ± 4	0 ± 0
3	196 ± 4	0 ± 0	240 ± 6*	2 ± 0.5*	386 ± 8*#	5 ± 1*	315 ± 3*#+	10 ± 2*#+
5	0	63 ± 7	125 ± 5*	71 ± 6	65 ± 3*#	47 ± 6	127 ± 4*+	82 ± 11+
7	0	64 ± 5	13 ± 1*	95 ± 12	17 ± 1*	90 ± 6	13 ± 1*	81 ± 8
14	0	0	0	1 ± 0.5*	0	3 ± 1*	0	3 ± 0.5*
21	0	0	0	0	0	0	0	0

The number of infective larvae were counted in the lungs cut in small fragments and put in greenhouse by 2 h to 27 °C, right away were centrifuged and the pellet examined for detection of the larvae using OM × 100. To count parasite worms the duodena were open longitudinal in saline solution and put in formaldehyde 10% and carried out histological cuts of 4 µm and H&E-stained and examined in the OM × 100. The data were express as mean ± SEM of six mice.

The values found are considered statistically significant.

Sv+H_2O vs. Sv+indomethacin, or Sv+H_2O vs. Sv+ibuprofen, or Sv+H_2O vs. Sv+celecoxib; #Sv+indomethacin vs. Sv+ibuprofen, or Sv+indomethacin vs. Sv+celecoxib; +Sv+ibuprofen vs. Sv+celecoxib. $^{*\#+}P < 0.05$.

Sv, *Strongyloides venezuelensis*.

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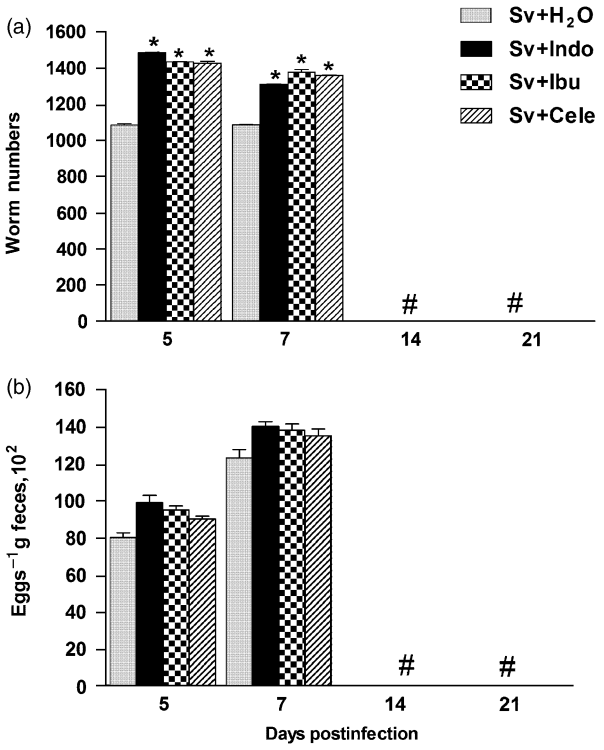

Fig. 2. Number of adult worms (a) in duodena eggs g^{-1} of feces (b) from *S. venezuelensis* (Sv)-infected mice, treated with indomethacin (Indo), ibuprofen (Ibu), celecoxib (Cele), or vehicle (H_2O). Data are expressed as mean ± SEM of two independent experiments ($n = 6$ per day). $^*P < 0.05$, *Sv+H_2O vs. Sv+Indo, or Sv+Ibu, or Sv+Cele.

(and mean number) of eggs recovered from the feces on days 5 and 7 postinfection in the treated animals were 24% ($99 \pm 9 \times 10^2$) and 14% ($140 \pm 6 \times 10^2$) for indomethacin, 19% ($195 \pm 5 \times 10^2$) and 4% ($128 \pm 8 \times 10^2$) for ibuprofen, and 10% ($88 \pm 4 \times 10^2$) and 7% (and $131 \pm 9 \times 10^2$) for celecoxib, respectively. For mice infected, but untreated, there were $80 \pm 6 \times 10^2$ and $123 \pm 10 \times 10^2$ eggs g^{-1} of feces on days 5 and 7, respectively. No eggs were detected in the feces of treated or untreated mice at other time periods (Fig. 2b). The eggs eliminated by the parasitic worms from cyclooxygenase inhibitor-exposed or untreated mice were fertile, as indicated by the emergence of infective larvae from cultures of these eggs.

Histopathological findings in the duodena and lung confirm the quantitative results

Figures 3 and 4 show histopathological analyses of the duodena and lungs of mice infected and treated with cyclooxygenase inhibitors or vehicle. On the third day postinfection, we observed worms located deep within the lamina propria and in the intestinal lumen in cyclooxygenase inhibitor-treated animals, whereas in untreated mice, parasites were not observed at this early time point following infection (Fig. 3a).

The number of parasitic females in the mucous membrane of the small intestine in the cyclooxygenase inhibitor-treated mice increased on days 5 and 7 postinfection and was maintained until day 14, as shown for indomethacin (Fig. 3f–h), ibuprofen (Fig. 3j–l), and celecoxib (Fig. 3n–p). In untreated mice, adult worms were accompanied by intense cellular infiltration in the lamina propria of the villi, with the obvious presence of eosinophils (Fig. 3b–d). Eosinophilic cell infiltration was also found in the small intestine of the cyclooxygenase inhibitor-treated mice, however, in less quantity. The number of inflammatory cells on day 14 postinfection remained stable in the untreated mice and the adult worms were completely expelled at this point of the infection (Fig. 3d). However, in the cyclooxygenase inhibitor-exposed mice, worms remained in the host for a longer duration of time and these females were both fertile and viable (Fig. 3h, m, and q).

Figure 4 shows the histopathological analyses of the lungs of infected mice treated with cyclooxygenase inhibitors. On day 3 postinfection, we observed larvae in the pulmonary tissue (Fig. 4e, i, and m), which were not seen in untreated, infected mice (Fig. 4a). The lungs of infected mice treated with indomethacin (Fig. 4f–h), ibuprofen (Fig. 4j–l), or celecoxib (Fig. 4n–p) demonstrated pulmonary hemorrhage until day 7 postinfection, while in the untreated mice, hemorrhagic foci were observed to only a small degree on days 3 and 5 (Fig. 4a and b).

In the untreated, infected mice, the passage of larvae through the lungs was accompanied by intense and diffuse inflammatory infiltrates with neutrophils, eosinophils, and hemosiderin-laden macrophages (Fig. 4b–d). Similar changes were seen in the lungs of cyclooxygenase inhibitor-treated mice, but to a lesser extent as shown for indomethacin (Fig. 4f–h), ibuprofen (Fig. 4j–l), and celecoxib (Fig. 4n–p). We did not observe any differences in the number of inflammatory cells on or after day 14 of infection in treated or untreated mice (Fig. 4d, h, l, and p).

Prostaglandin inhibitors decrease eosinophils in the blood, peritoneal cavity, and bronchoalveolar space during *S. venezuelensis* infection

Figure 5a shows that infection with *S. venezuelensis* induced significant increases ($P < 0.05$) in the total leukocytes in the blood from day 5 of infection in comparison with the group of uninfected mice, with a peak on day 14 postinfection. The treatment of infected mice with cyclooxygenase inhibitors did not alter the total number of circulating cells compared with untreated, infected mice.

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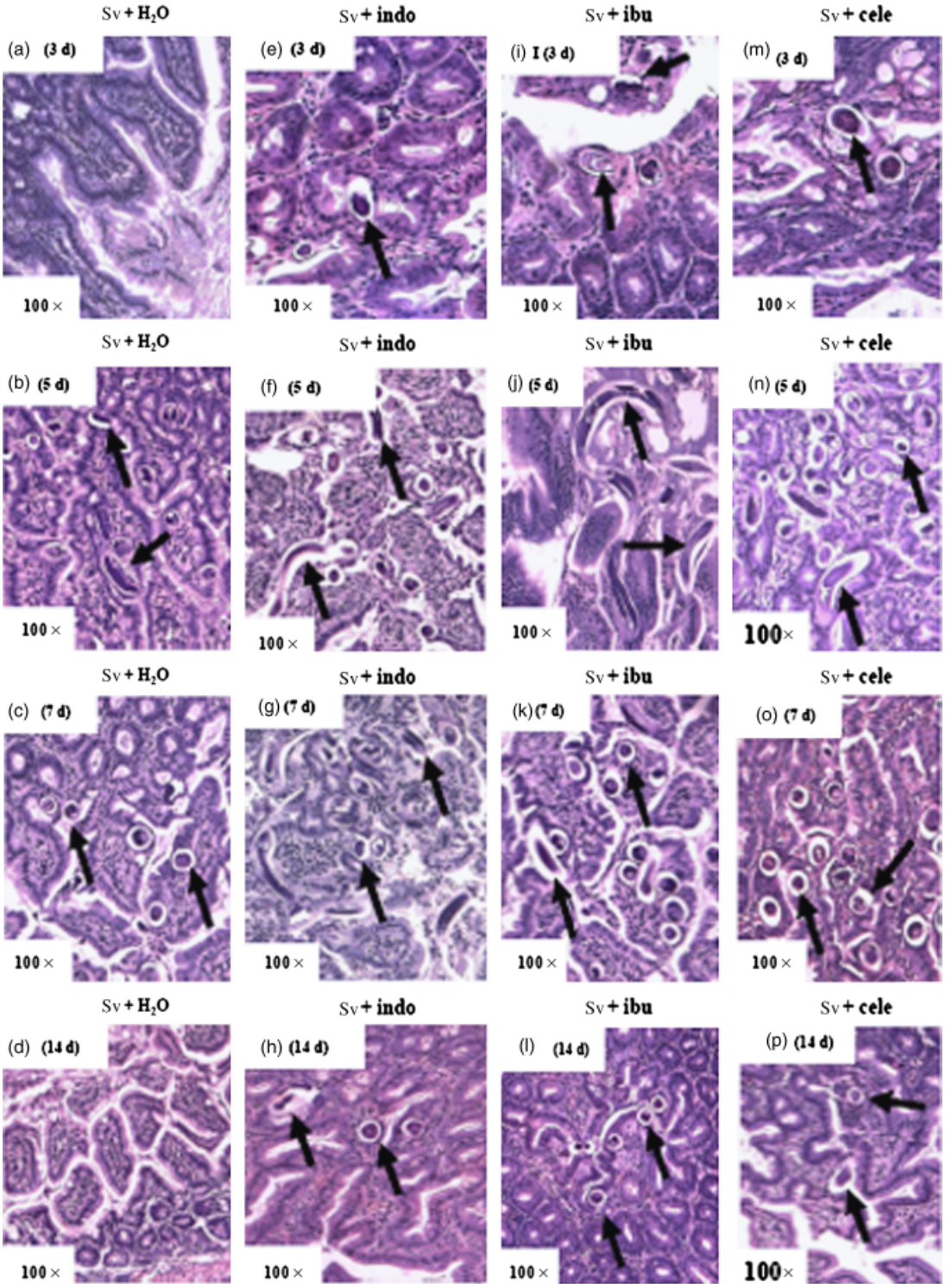

Fig. 3. Histopathology of duodena from *S. venezuelensis* (Sv)-infected Swiss mice. Infected Swiss mice were treated orally daily with vehicle (H_2O), indomethacin (Indo), ibuprofen (Ibu), or celecoxib (Cele). Representative H&E-stained duodenal sections from Sv+H_2O (a–d), Sv+Indo (e–h), Sv+Ibu (i–l), and Sv+Cele (m–p). The arrows indicate sections with adult worms. Magnification of panels × 100.

The recruitment of total leukocytes into the peritoneal cavity (Fig. 5b) showed a time-dependent increase in cells during the course of infection compared with uninfected rodents. This increase was significant in all of the periods analyzed and peaked on day 14 postinfection. The treatment of mice with indomethacin did not interfere with the

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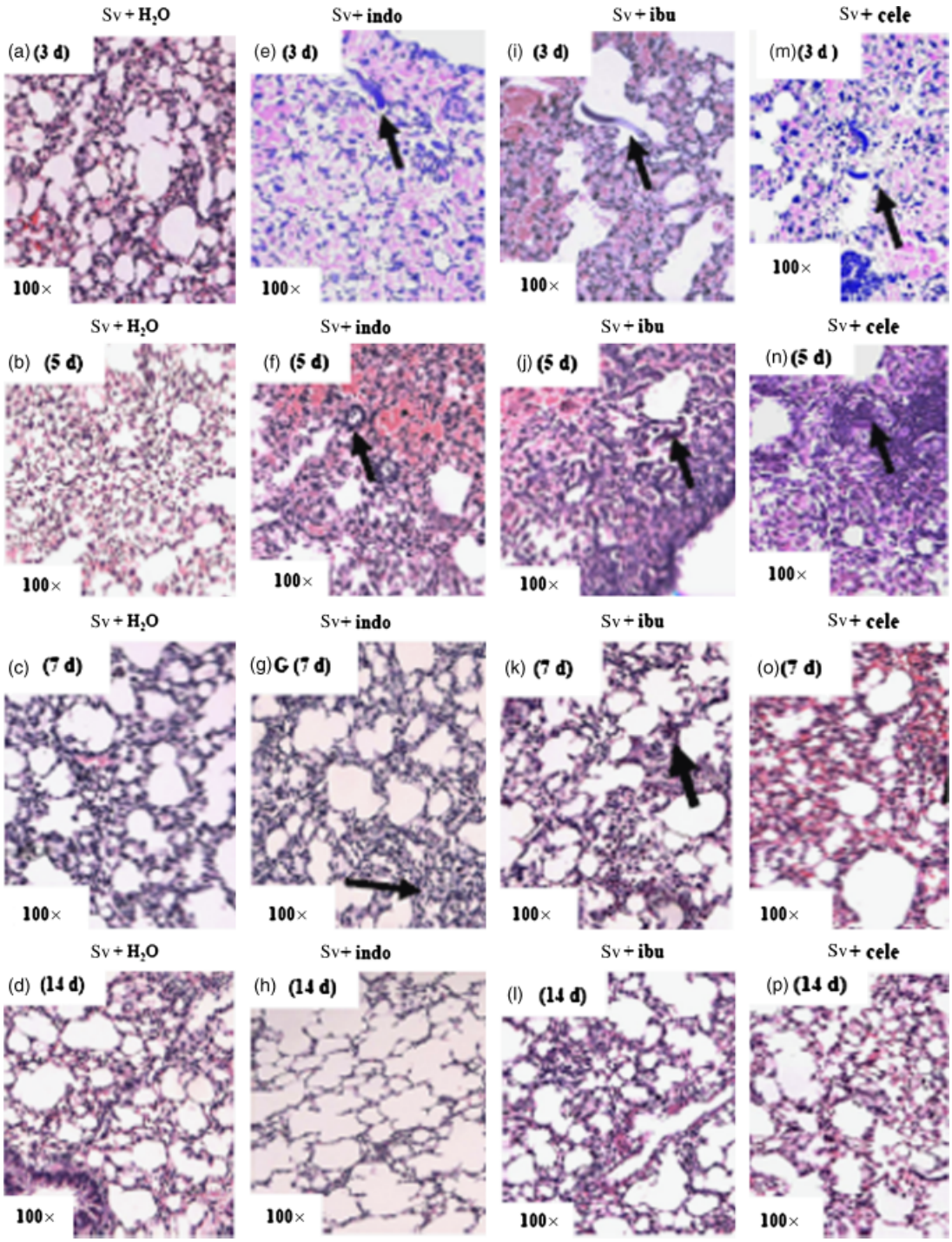

Fig. 4. Histopathology of lungs from *S. venezuelensis* (Sv)-infected Swiss mice. Infected Swiss mice were treated orally daily with vehicle (H_2O), indomethacin (Indo), ibuprofen (Ibu), or celecoxib (Cele). Representative H&E-stained duodenal sections from Sv+H_2O (a–d), Sv+Indo (e–h), Sv+Ibu (i–l), and Sv+Cele (m–p). The arrows indicate sections with adult worms. Magnification of panels × 100.

migration of the total leukocytes into the PCF when compared with untreated, infected mice. However, when mice were treated with ibuprofen or celecoxib, we observed a small reduction in the total number of PCF leukocytes, with a significant reduction compared with untreated, infected mice only seen on day 14. The total leukocyte counts in the PCF of mice treated with ibuprofen were significantly lower than those observed in mice given indomethacin only on

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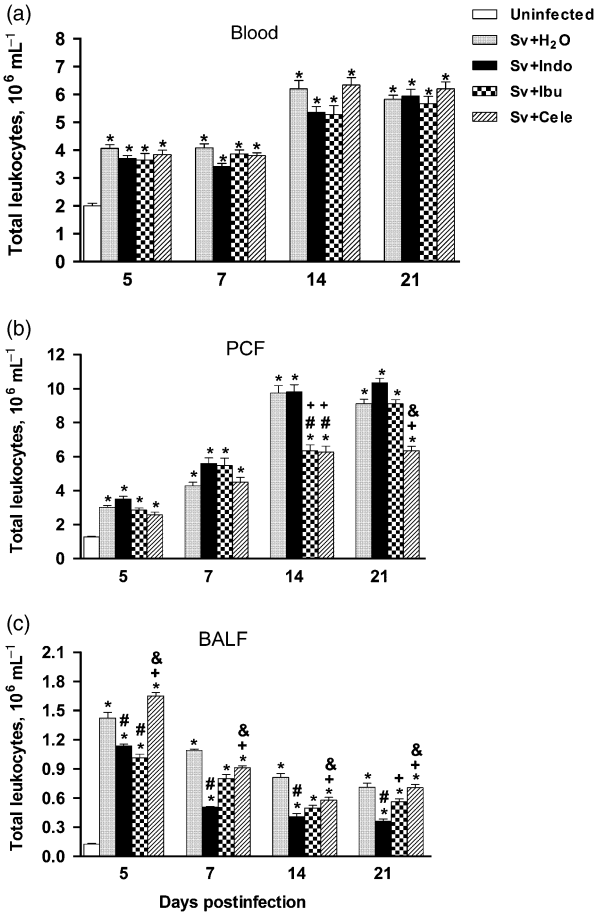

Fig. 5. Total leukocytes in blood, PCF, and BALF. Cells were obtained from Swiss mice postinfection with *S. venezuelensis* (Sv) larvae, treated orally daily with vehicle (H_2O), indomethacin (Indo), ibuprofen (Ibu), or celecoxib (Cele). A group of uninfected mice was used as a control. Numbers of total leukocytes (a–c) were enumerated and identified through Rosenfeld staining. Data are expressed as mean ± SEM of two independent experiments ($n = 6$–8 per day) and uninfected mice ($n = 5$–6 per day). $^{*\#+\&}P < 0.05$. *Uninfected control mice vs. Sv+H_2O, or Sv+Indo, or Sv+Ibu, or Sv+Cele; #Sv+H_2O vs. Sv+Indo, or Sv+Ibu, or Sv+Cele; +Sv+Indo vs. Sv+Ibu or Sv+Cele; &Sv+Ibu vs. Sv+Cele.

day 14 or given celecoxib on days 14 and 21. Significant differences between treatment with either ibuprofen or celecoxib were observed on day 21 postinfection (Fig. 5b).

Infection of mice with *S. venezuelensis* also induced significant migration of total leukocytes into the bronchoalveolar space (Fig. 5c), with a peak on day 5 of infection. The treatment of the infected mice with indomethacin significantly reduced the number of leuckocytes in the BALF compared with untreated, infected mice on day 5 postinfection. Such a difference was not seen with ibuprofen or celecoxib after day 7 of infection. In fact, indomethacin inhibited the migration of leukocytes into this compartment, compared with ibuprofen or celecoxib, at every day analyzed following infection (Fig. 5c).

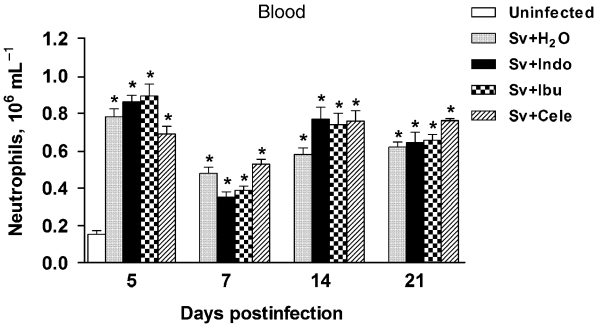

Fig. 6. Neutrophils numbers in blood. Cells were obtained from Swiss mice postinfection with *S. venezuelensis* (Sv) larvae and treated orally daily with vehicle (H_2O), indomethacin (Indo), ibuprofen (Ibu), or celecoxib (Cele). A group of uninfected mice was used as a control. Numbers of neutrophils were enumerated and identified through Rosenfeld staining. Data are expressed as mean ± SEM of two independent experiments ($n = 6$–8 per day) and uninfected mice ($n = 5$–6 per day). $^{*}P < 0.05$. *Uninfected control mice vs. Sv+H_2O, or Sv+Indo, or Sv+Ibu, or Sv+Cele.

Mice infected with larvae of *S. venezuelensis* presented significant increases of neutrophils in the blood at all periods analyzed compared with uninfected mice. The treatment of mice infected with cyclooxygenase inhibitors did not affect the number of circulating neutrophils in comparison with untreated, infected animals. In all four groups of infected mice, there was a peak in the increase of neutrophils on day 5 postinfection (Fig. 6). Neutrophils were not present in the peritoneal cavity and in the bronchoalveolar space (data not shown).

Increases in circulating blood eosinophils in response to *S. venezuelensis* larvae were time dependent (Fig. 7a), with a peak on day 14 postinfection. The treatment of infected mice with cyclooxygenase inhibitors significantly reduced the number of eosinophils in the blood when compared with untreated, infected mice. Indomethacin significantly decreased the number of these cells between days 5 and 14 of the infection compared with ibuprofen or celecoxib treatment (Fig. 7a).

The increases in mononuclear cells in the blood and the influx of these cells into the peritoneal cavity and bronchoalveolar space in response to infection with *S. venezuelensis* are shown (Fig. 7d–f). The number of mononuclear cells in the blood of the infected animals was significantly higher than in the uninfected mice in all of the periods analyzed, presenting two peaks on days 14 and 21 postinfection. The treatment of the mice infected with cyclooxygenase inhibitors did not inhibit the number of those cells in the blood (Fig. 7d).

The kinetics of recruitment of eosinophils into the peritoneal cavity of infected mice was also time dependent and peaked on day 21 postinfection (Fig. 7b). In the mice

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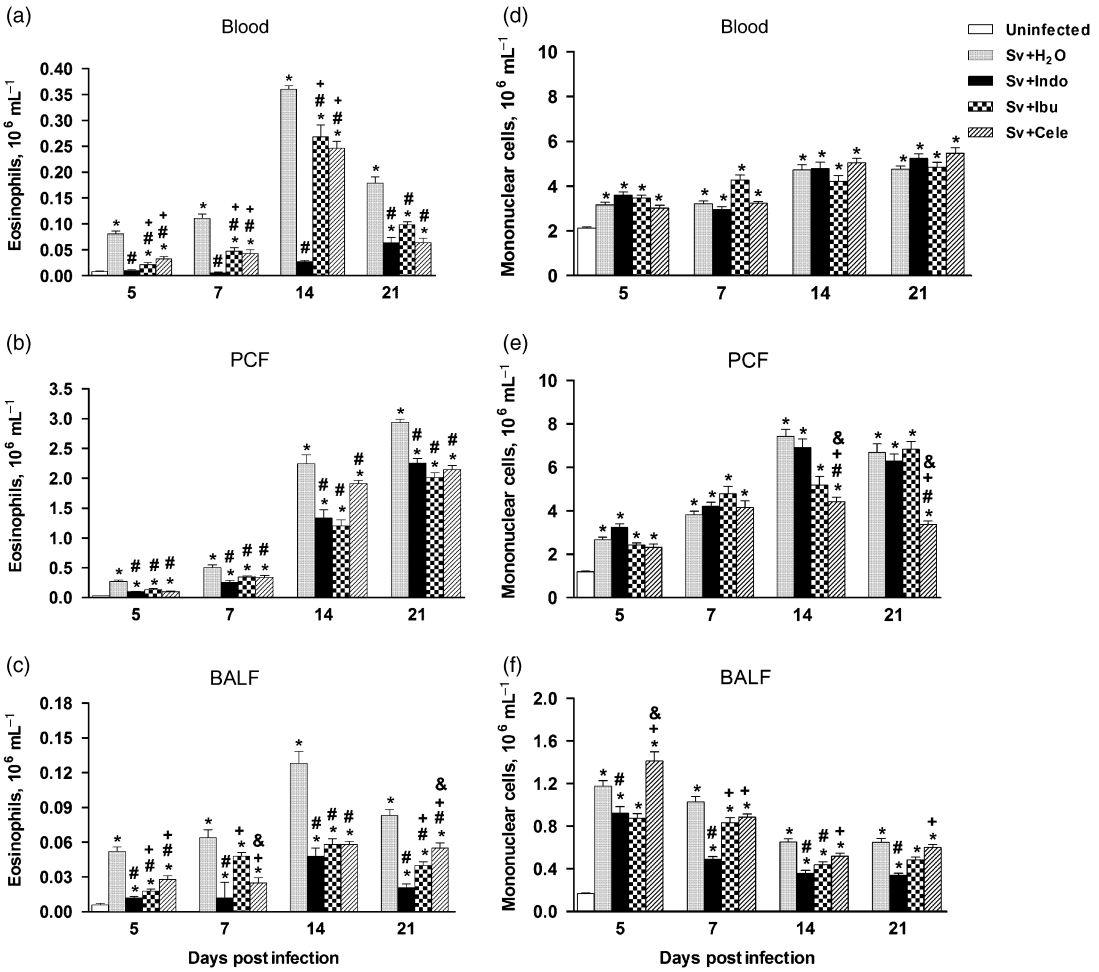

Fig. 7. Eosinophils and mononuclear cells in blood, PCF, and BALF. Cells were obtained from Swiss mice postinfection with *S. venezuelensis* (Sv) larvae and treated orally daily with vehicle (H_2O), indomethacin (Indo), ibuprofen (Ibu), or celecoxib (Cele). A group of uninfected mice was used as a control. Numbers of eosinophils (a–c) and mononuclear cells (d–f) were enumerated and identified through Rosenfeld staining. Data are expressed as mean ± SEM of two independent experiments ($n = 6$–8 per day) and uninfected mice ($n = 5$–6 per day). $^{*\#+\&}P < 0.05$. *Uninfected control mice vs. Sv+H_2O, or Sv+Indo, or Sv+Ibu, or Sv+Cele; $^{\#}$Sv+H_2O vs. Sv+Indo, or Sv+Ibu, or Sv+Cele; $^{+}$Sv+Indo vs. Sv+Ibu or Sv+Cele; $^{\&}$Sv+Ibu vs. Sv+Cele.

infected and treated with cyclooxygenase inhibitors, the numbers of eosinophils decreased significantly between days 5 and 21 postinfection, when compared with untreated and infected mice. We observed significant increases of mononuclear cells in the PCF soon after the beginning of infection, in comparison with uninfected mice (Fig. 7e). We also observed two peaks in the migration of these cells on days 14 and 21 postinfection. Indomethacin and ibuprofen did not alter the influx of these cells into this compartment compared with untreated mice. On the other hand, treatment with celecoxib inhibited the recruitment of the mononuclear cells into the peritoneal cavity in the periods analyzed, but significant differences were observed only on days 14 and 21 postinfection (Fig. 7e).

In the bronchoalveolar space of infected mice, eosinophilia was significant on day 5 postinfection and peaked on day 14. The treatment with indomethacin inhibited the recruitment of eosinophils on every day analyzed, with significant differences compared with untreated, infected mice between days 5 and 21 postinfection (Fig. 7c). The treatment of animals with ibuprofen and celecoxib also inhibited the influx of eosinophils into the bronchoalveolar space; significant differences were observed in these infected mice compared with untreated, infected mice across all days analyzed, except day 7. Of the three drugs used in the treatment, indomethacin decreased the influx of eosinophils into the bronchoalveolar space most potently.

The recruitment of mononuclear cells into the bronchoalveolar space from infected mice (Fig. 7f) was significantly higher than that in uninfected mice after day 5 of infection, with a peak that day and progressive decreases through day 21. The treatment of mice with indomethacin

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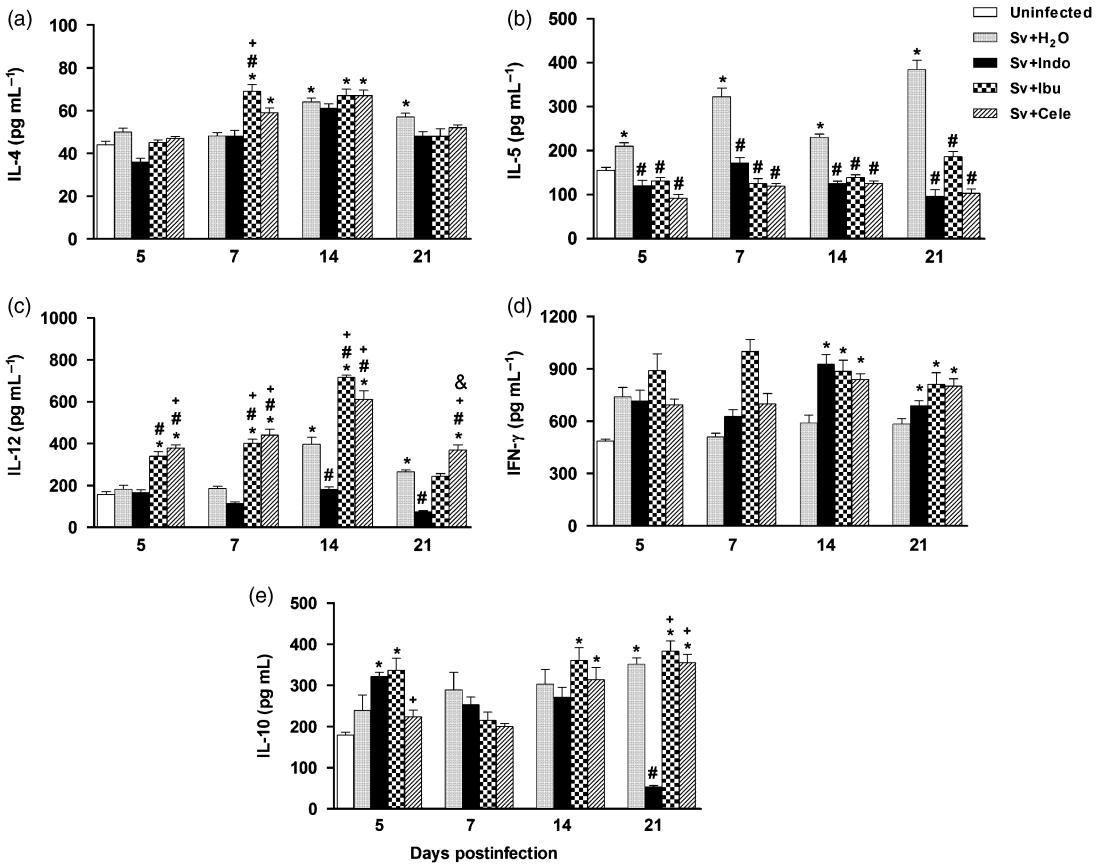

Fig. 8. Cytokine levels in lung tissue. Mice were subjected to infection with *S. venezuelensis* (Sv) larvae and daily treated orally daily with vehicle (H_2O), indomethacin (Indo), ibuprofen (Ibu), or celecoxib (Cele). A group of uninfected mice was used as a control. Cytokine levels were determined by ELISA. Data are expressed as mean ± SEM of two independent experiments ($n = 6$–8 per day) and uninfected mice ($n = 5$–6 per day). $^{*\#+\&}P < 0.05$. *Uninfected control mice vs. Sv+H_2O, or Sv+Indo, or Sv+Ibu, or Sv+Cele; $^{\#}$Sv+H_2O vs. Sv+Indo, or Sv+Ibu, or Sv+Cele; $^{+}$Sv+Indo vs. Sv+Ibu or Sv+Cele; $^{\&}$Sv+Ibu vs. Sv+Cele.

significantly inhibited the influx of mononuclear cells into the bronchoalveolar space in all days postinfection when compared with either untreated mice or celecoxib-treated infected animals. In animals treated with ibuprofen, the number of mononuclear cells was significantly lower compared with untreated, infected animals only on day 14. In the lungs, the treatment with celecoxib did not alter the kinetics of the mononuclear cells in any of the periods analyzed, except on day 5, when mononuclear cells were significantly higher compared with animals treated with indomethacin or ibuprofen.

Prostaglandin inhibitors modulate the synthesis of cytokines in mice infected with *S. venezuelensis*

The profile of the cytokines IL-4, IL-5, IL-10, IL-12, and IFN-γ in the homogenized lung tissue of mice infected with *S. venezuelensis* larvae treated with cyclooxygenase inhibitors (or vehicle) is presented in Fig. 8. The levels of IL-4 in infected mice were significantly higher on days 14 and 21 postinfection compared with uninfected mice (Fig. 8a). Interestingly, the treatment of mice with indomethacin inhibited the synthesis of this cytokine only from day 5 postinfection, being lower than or similar to the values found in uninfected mice. However, treatment with ibuprofen or celecoxib did not inhibit the synthesis of IL-4, which was similar to uninfected mice across all times analyzed.

The infection with *S. venezuelensis* induced significant increases in the synthesis of IL-5 between days 5 and 21 postinfection (Fig. 8b). The treatment of mice with cyclooxygenase inhibitors inhibited the synthesis of IL-5 during all of the days evaluated when compared with the mice only infected. The concentration of IL-12 (Fig. 8c) was increased in the mice infected in every day analyzed; however, it was significant compared with uninfected mice only on days 14 and 21 postinfection. In mice infected and treated with indomethacin, the synthesis of this cytokine was inhibited on every day analyzed when compared with untreated, infected mice, with statistical significance observed on days

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14 and 21 postinfection. Unexpectedly, the levels of IL-12 were significantly enhanced with ibuprofen and celecoxib (Fig. 8c), particularly at later time points.

The level of IFN-γ (Fig. 8d) in response to infection varied over the periods analyzed. The production of this cytokine was increased on day 5 postinfection. The results obtained from cyclooxygenase inhibitor-treated mice were similar to untreated mice on days 5 and 7. However, on days 14 and 21, these three drugs induced significant increases of this cytokine when compared with uninfected mice.

The synthesis of IL-10 in the infected animals (Fig. 8e) varied among the days analyzed, being significantly higher on day 21 postinfection compared with uninfected mice. Indomethacin had little effect on IL-10 synthesis, except on day 21 postinfection, where the drug significantly suppressed IL-10 generation compared with untreated, infected animals. Ibuprofen had no effect on IL-10 compared with vehicle alone; however, the levels were higher than those in uninfected animals. Celecoxib treatment impaired IL-10 generation only on day 7 following infection.

Prostaglandin inhibitors modulate the synthesis of antibodies in mice infected with *S. venezuelensis*

The parasite-specific IgG1, IgG2a and IgE levels were quantified in the serum of mice sacrificed on days 7, 14, and 21 postinfection (Fig. 9). The concentration of IgG1 was significantly higher in the infected mice, compared with the uninfected mice, over the 3 days analyzed (Fig. 9a). The treatment with cyclooxygenase inhibitors significantly decreased the synthesis of IgG1 compared with untreated, infected mice. Only indomethacin significantly decreased the levels of IgG2a (on day 21 postinfection; Fig. 9b).

The synthesis of IgE (Fig. 9c) in the infected mice showed significantly increased values on days 7, 14, and 21 postinfection when compared with uninfected mice. Treatment of mice with cyclooxygenase inhibitors significantly decreased the synthesis of IgE when compared with untreated, infected mice.

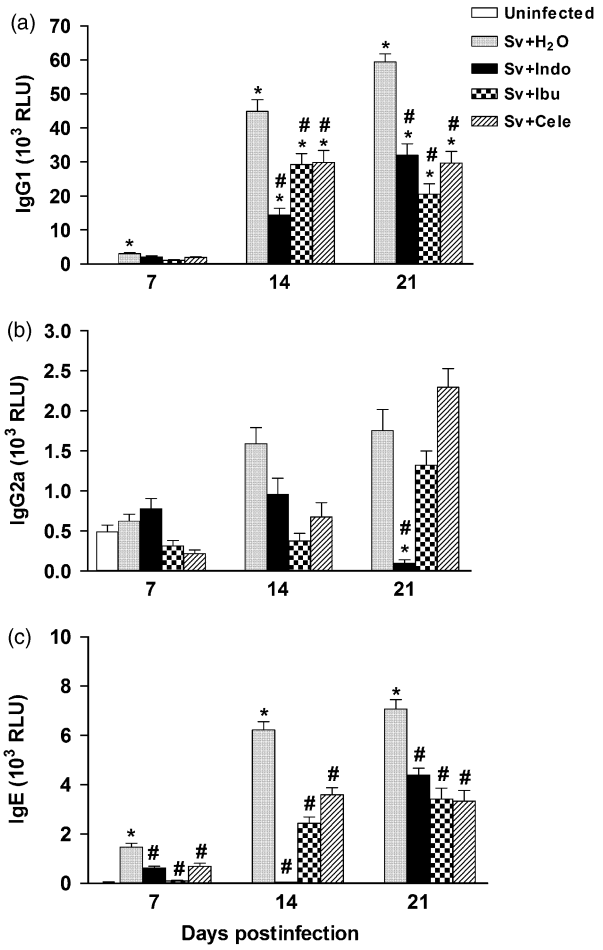

Fig. 9. Specific antibodies IgG1 (a), IgG2a (b), and IgE (c) in the serum of *S. venezuelensis* (Sv)-infected mice, treated orally daily with vehicle (H_2O), indomethacin (Indo), ibuprofen (Ibu), or celecoxib (Cele). A group of uninfected mice was used as a control. Antibodies were determined by ELISA and developed by chemiluminescence. The data are expressed as mean ± SEM of relative light units (RLU) of two independent experiments ($n = 6$–8 per day) and uninfected mice ($n = 5$–6 per day). $^{*\#}P < 0.05$. *Uninfected control mice vs. Sv+H_2O, or Sv+Indo, or Sv+Ibu, or Sv+Cele; #Sv+H_2O vs. Sv+Indo, or Sv+Ibu, or Sv+Cele.

Discussion

In the present study, we reveal an important role for prostaglandins in regulating the immune responses of mice infected with *S. venezuelensis*, a model for human infection with *S. stercoralis*. As described previously, infection of mice with *S. venezuelensis* induced increases in the total leukocyte numbers, neutrophils, eosinophils, and mononuclear cells in the blood, peritoneal cavity, and bronchoalveolar space (Machado *et al.*, 2005), with each cell type demonstrating a different kinetic profile in these various biological compartments. The treatment of infected mice with cyclooxygenase inhibitors partially inhibited the increase of neutrophils, eosinophils, and mononuclear cells in the blood and the recruitment of these cells into the peritoneal cavity and bronchoalveolar space. Among the three myeloid lineages, eosinophils were the most regulated by cyclooxygenase inhibition, suggesting that in this model of infection, prostaglandins regulate eosinophil numbers and trafficking more than neutrophils or monocytes. Of the three cyclooxygenase inhibitors used, indomethacin was the most potent inhibitor of eosinophil responses to infection. The basis for this pharmacological difference remains unknown. Whether the unique effects of indomethacin can be attributed to off-target effects such as ligation of the CRTH2 receptor for PGD_2 or the activation of the PPAR-γ nuclear receptor remains speculative (Hirai *et al.*, 2002).

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The results in the present experimental model of *S. venezuelensis* suggest that prostaglandins are inflammatory mediators of great importance to the host during strongyloidiasis. Although we now show that PGE_2 levels increase during infection and are partially blocked by cyclooxygenase inhibitors, it remains unclear whether PGE_2 causally mediates the immune responses to *Strongyloides per se*. Our data using cyclooxygenase inhibitors suggest that multiple downstream cyclooxygenase-derived prostaglandins might be playing a role, including PGI_2, TXA_2, and/or PGD_2. A limitation of this study is that we did not quantify prostaglandins other than PGE_2. This may be relevant as PGD_2 (for example) is involved in the trafficking of Th2 cells, eosinophils, neutrophils, and mononuclear cells to inflammatory sites in eosinophilic/parasitic/allergic diseases (Chevalier *et al.*, 2005; Satoh *et al.*, 2006; Schratl *et al.*, 2007). In addition, PGD_2 was found to play a protective role in bacterial pneumonia (Joo *et al.*, 2007). Future experiments to define the immunoregulatory roles of other downstream cyclooxygenase metabolites of AA during strongyloidiasis are necessary. To define specific roles for PGE_2 in *Strongyloides* infections, future experiments might include the infection of EP receptor knockout mice; the use of selective EP receptor antagonists; or the infection of PGE_2 synthesis null mice. Another interesting point requiring further attention in light of the present study is the potential involvement of parasite-derived prostaglandins in the pathogenesis of *Strongyloides* infections. Other parasites, such as *S. mansoni* (Angeli *et al.*, 2001) and *Trypanosoma brucei* (Kubata *et al.*, 2000), have been shown to synthesize prostaglandins that affect host immune responses. Whether any effects of cyclooxygenase inhibitors seen in our study reflect changes in parasite-derived prostaglandins remains unknown.

The prostaglandins are inhibited by glucocorticoids and nonsteroidal anti-inflammatory drugs such as indomethacin, aspirin, meloxicam, diclofenac, celecoxib, and ibuprofen (Ramaswamy *et al.*, 2000; López-Armada *et al.*, 2002; Süleyman *et al.*, 2004). Indomethacin has often been used in studies of the involvement of prostaglandins in the inflammation induced by parasites (Daugschies, 1995; Rakha *et al.*, 1996; Daugschies & Ruttkowski, 1998; Terrazas *et al.*, 1999; Nishina & Suzuki, 2002). Ibuprofen is an anti-inflammatory drug that inhibits the differentiation and proliferation of inflammatory cells, increases apoptosis, induces the death of bacteria, and reduces the fertility of helminths (Farag *et al.*, 1995; Hockertz & Heckenberger, 1996; Yoon *et al.*, 2003; Hanna *et al.*, 2004). Celecoxib has been approved recently in the treatment of the arthritis rheumatoid and osteoarthritis (Everts *et al.*, 2000). *In vivo*, celecoxib suppressed the development of cancer in the colon, lungs, head, and neck (Williams *et al.*, 2000; Rao *et al.*, 2002; Zweifel *et al.*, 2002; Mima *et al.*, 2005) and increased the antitumoral activity of chemotherapeutic agents (Trifan *et al.*, 2002).

Infection by *S. venezuelensis* increased the synthesis of cytokines of the Th2 pattern such as IL-4 and IL-5 in all of the time points analyzed, and IL-10 mainly on days 1, 3, and 21. In contrast, on later days postinfection, the Th1 cytokine IL-12 was increased. These results are similar to the data reported in the literature, showing that *Strongyloides* sp. induced immune responses accompanied by an increase of the production of cytokines and antibodies mainly of the Th2 pattern (Korenaga *et al.*, 1991; Herbert *et al.*, 2002; Machado *et al.*, 2005), although Th1 cytokines are produced (Kuroda *et al.*, 2001; Machado *et al.*, 2005). The cytokine IL-12 typically amplifies cytokine production along a Th1 pattern (e.g. IFN-γ), which confers protection to the host by activating infected cells. For example, spleen cells cultured from BALB/c mice treated with indomethacin and infected with the intracellular parasites *Leishmania mexicana* and *Leishmania major* showed increased IL-12 synthesis and diminished growth and replication of the microorganisms (Perez-Santos & Talamas-Rohana, 2001). In contrast, our previous results demonstrated that IL-12 suppresses Th2-type protective immunity, increasing the survival and persistence of parasites in the intestine of an *S. venezuelensis*-infected host (Machado *et al.*, 2009). Thus, IL-12 appears to have protective effects for the parasite, which may be counterbalanced by the Th2 mediators IL-4 and IL-5, because adult parasites were completely expelled after the 14th day postinfection in mice lacking IL-12 (Machado *et al.*, 2009). These data agree with earlier reports that IL-12 favors parasitism (Rotman *et al.*, 1997).

The treatment of animals with indomethacin inhibited IL-4, IL-5, IL-10, and IL-12 synthesis on different days postinfection (Fig. 8a–c and e). The results obtained from IL-4 and IL-10 levels corroborate the findings presented in the literature, where several authors have shown that indomethacin administrated *in vitro* or *in vivo* in *S. mansoni* or *Taenia crassiceps* infections inhibited the synthesis of these cytokines (Tsuboi *et al.*, 1995; Terrazas *et al.*, 1999; Ramaswamy *et al.*, 2000). Ibuprofen and celecoxib inhibited IL-5 synthesis and increased IL-12 production. In the present studies, treatment with indomethacin, but not ibuprofen or celecoxib, inhibited IL-12 production, but also contributed to the retention of high worm numbers and prolonged the time of the parasitism in the host. These results emphasize that the importance of Th1 and Th2 immunity to host defense is dependent on context and both arms of adaptive immunity have evolved for protective purposes.

It is well demonstrated that eosinophils contribute to the elimination of *S. venezuelensis* (El-Malky *et al.*, 2003) and *S. stercoralis* in mice (Herbert *et al.*, 2000), and that protective immunity against larvae migrating in deep tissue was dependent on IL-5 (Korenaga *et al.*, 1991). In addition, data described in the literature show that IL-4, even in small

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concentrations, is able to induce the synthesis of IgG and IgE (Finkelman *et al.*, 1988). These antibodies are involved in the cytotoxicity mediated by eosinophils or antibody-dependent cellular cytotoxicity (ADCC) (Van Milligen *et al.* 1998), and also contribute in the degranulation of mast cells (Urban *et al.*, 2001), leading, as a consequence, to the expulsion and death of the parasites. In our study, the synthesis of parasite-specific antibodies was also decreased by cyclooxygenase inhibitors, and the low levels of antibodies seem to contribute to the deficiency in ADCC.

In conclusion, here, we reveal a previously unknown contribution of prostaglandins to immune responses evoked in response to infection by *S. venezuelensis*. The inhibition of prostaglandin synthesis resulted in increases of parasite burden, but did not interfere directly with female fertility, suggesting that the regulation of host defense mechanisms mediated by prostaglandins is important in the control of infection. Our results show that these lipid mediators contribute to eosinophil and mononuclear cell migration, modulate Th2 cytokines, such as IL-4, IL-5, IL-10, and regulate specific-parasite antibodies IgG1 and IgE. These prostaglandin-regulated mechanisms are crucial to the protection of the host against strongyloidiasis. Our findings hold important implications for the development of novel preventive or therapeutic agents against human *Strongyloides* infections.

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