

Interleukin-5 modulates interleukin-8 secretion in eosinophilic inflammation

L. H. Faccioli,^{1,CA} A. I. Medeiros,¹ A. Malheiro,¹
R. C. L. R. Pietro,¹ A. Januário¹ and B. B. Vargaftig²

¹Department of Parasitology, Microbiology and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil;

²Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur/INSERM n. 285, Paris, France

^{CA}Corresponding Author

Fax: (+55) 16 633 6631

Email: facciol@fmrp.usp.br

SERUM and BALF (bronchoalveolar lavage fluid) IL-8 levels and serum levels were investigated in *Toxocara canis* infected guinea-pigs and the role of IL-5 as a modulator of cytokine secretion was studied. Serum levels increased early in infected animals, exceeding control levels 4 h after infection, peaked between days 6 and 18, and continued to exceed control levels after 48 days of infection. Serum and BALF IL-8 levels showed the same profile as blood eosinophilia, increasing 6 days post-infection and peaking between days 18 and 24. Treatment of infected animals with anti-IL-5 Ab suppressed eosinophilia with a parallel increase in blood IL-8 levels, whereas no change was found in levels. To support our *in vivo* observation we carried out experiments *in vitro* using guinea-pig LPS-stimulated adherent peritoneal cells which release large amounts of IL-8 into the supernatants. When rIL-5 was added to LPS-stimulated cells, 65% inhibition of IL-8 release into the supernatants was observed. Pre-incubation of cells with anti-IL-5 Ab prevented the inhibition of IL-8 release into the supernatants induced by rIL-5. Our results demonstrate for the first time that TNF- α and IL-8 are released concomitant with or after IL-5 in the eosinophilic inflammation induced by *T. canis*. Moreover, in addition to showing that IL-5 is fundamental for the induction of blood eosinophilia, the present results suggest that this cytokine may play a new biological role by acting as modulator of IL-8 secretion.

Key words: Interleukins, Eosinophils, Inflammation, *Toxocara canis*

Introduction

Eosinophilia has been associated with parasitic diseases, particularly when the parasites invade the tissue or injure the mucosal surfaces.¹ *Toxocara canis* is an intestinal parasite of dogs and is the most common aetiologic agent of visceral larva migrans syndrome in humans. Beaver *et al.*,² when describing this syndrome, noted intense eosinophilia which reached more than 90% of total leukocyte counts. Despite previous observations that IL-5 participates in *T. canis*-induced eosinophilia³ and drives eosinophils from the bone marrow to blood,³ little is known about the presence of other cytokines related to eosinophil recruitment and the physiological control of blood eosinophilia in this parasitic model.

Using the well established guinea-pig model of *T. canis* infection,³ we determined the release of two relevant cytokines, TNF- α and IL-8, and studied their interaction with IL-5. This cytokine is an inducible glycoprotein produced and secreted by T cells, mast cells and eosinophils,⁴ which promotes the differentiation and proliferation of eosinophils⁵ and plays an essential role in the induction of eosinophilia in

allergic inflammation,⁶ asthma,⁷ viruses,⁸ and helminthic infection.^{3,9,10} Anti-IL-5 Ab treatment inhibited blood and tissue eosinophilia.^{3,10,11} IL-8 is a member of the C-X-C chemokines described as neutrophil chemotactic factor¹² which also attracts eosinophils.^{13,14} IL-8 is synthesized by monocytes,¹² eosinophils,¹⁵ and other cells in response to several stimulus. IL-8 is an important mediator of lung disease¹⁶ and may be relevant in asthma.¹⁷ Finally, TNF- α is an important mediator of inflammation produced in response to various stimuli such as bacteria,¹⁸ fungi,¹⁹ and allergen challenge.²⁰ TNF- α is also involved in eosinophil recruitment^{21,22} and induces *in vivo* and *in vitro* IL-8 release.^{23,24}

In the present study we first investigated whether TNF- α and IL-8 are released during *T. canis*-induced eosinophilic inflammation in guinea-pigs. Since during the course of infection we observed a sequential release of TNF- α and IL-8 in connection with IL-5 release,³ we also examined the relationship between IL-5 levels and IL-8 and TNF- α release *in vivo* by inducing IL-5 depletion with anti-IL-5 Ab. To corroborate the results obtained *in vivo* we carried out experiments *in vitro* studying the effect of rIL-5 on IL-

8 release in supernatants of LPS-stimulated adherent peritoneal cells.

Material and Methods

Reagents, MAbs and cytokines

Human rIL-5 was a kind gift from Dr S. Poole, National Institute for Biological Standards and Control (NIBSB), UK. The rat MAb TRFK-5 was initially provided by Dr P. Minoprio, Institut Pasteur, Paris, and later raised and purified by Mrs M. A. Nahori (Institut Pasteur, Paris). Irrelevant antibody was rat IgG against total anti-human IgG. LPS from *E. coli* 0111:B4 was from Sigma (Sigma Chemical Co., St Louis, MO, USA) and thioglycollate medium was from Difco (USA). Cytokines were measured using commercially available ELISA kits (Quantikine—R & D System, Minneapolis, MN). The kits were sensitive to human IL-8 and TNF- α levels of 5 pg/ml and 4.8 pg/ml, respectively.

Animals

Outbred albino female guinea-pigs weighing 300–400 g were obtained from the University of São Paulo and kept in the animal house of the Department of Parasitology, Microbiology and Immunology, School of Medicine of Ribeirão Preto, under routine conditions.

Infection of animals

T. canis eggs were obtained from gravid female worms recovered from dogs. The eggs were removed from the uterus, washed and allowed to develop to the infective stage in shallow dishes containing 0.5% formalin at 37°C.³ Guinea-pigs were infected with 1 ml saline containing 500 *T. canis* eggs, by gastric intubation using a metal cannula, under light ether anaesthesia. Guinea-pigs receiving 1 ml of saline orally were used as control.

Blood cell determination and serum collection

Blood was obtained and cell counts were performed as previously described.³ Briefly, guinea-pigs were anaesthetized with sodium pentobarbitone (30 mg/kg, i.v.) and blood samples were collected by cardiac puncture. A sample was immediately added to a tube containing Turk solution and total cell counts were carried out using a Neubauer chamber. Another sample was used to prepare a smear, and differential counts were obtained using Rosenfeld-stained slices. Five ml of blood was allowed to coagulate and the serum separated and kept at -20°C.

Bronchoalveolar lavage fluid (BALF) and femur flushing liquid

Infected and non-infected guinea-pigs killed with overdose of sodium pentobarbitone and 5 ml of phosphate-buffered saline (PBS) containing 0.5% sodium citrate (PBS/SC) were instilled through a polyethylene cannula introduced into the trachea.³ The cell-free supernatants were recovered immediately and kept at -20°C. Femur flushing liquid was collected from guinea-pigs using 5 ml of PBS/SC, and cell free fluid was stored at -20°C.

BAL, femur flushing liquid and serum cytokine determinations

Serum, BALF and femur flushing fluid were used to measure IL-8-like and TNF- α like concentration using human ELISA kits.

TRFK-5 treatments *in vivo*

T. canis-infected guinea-pigs were injected i.p. with TRFK-5, an anti-IL-5 Ab, only once (2 mg/animal) at the time of infection (protocol A, PrA). Another group of infected animals received several i.p. anti-IL-5 Ab treatments at different intervals (1 mg/animal) at the time of infection followed by 0.3 mg/animal on days 1, 3, 12 and 17 thereafter (protocol B, PrB). A group of *T. canis* infected guinea-pigs was treated with irrelevant Ab using PrB. A group of infected guinea-pigs without treatment and a group of non infected animals were used for comparison. All animals were sacrificed 18 days after administration of *T. canis* eggs.

Preparation of adherent peritoneal cells and IL-8 generation *in vitro*

Peritoneal exudate cells were obtained from outbred female guinea-pigs (300 g) 4 days after an i.p. injection of 10 ml of 3% thioglycollate (Difco). Guinea-pigs were sacrificed with an overdose of sodium pentobarbitone and the cells were harvested from the peritoneal cavities by injection of 20 ml of RPMI 1640 (Gibco BRL, Grand Island) containing 10 U/ml of heparin without FCS. The peritoneal exudate consisted of 95–98% mononuclear cells (macrophages and lymphocytes) on the basis of morphology determined by light microscopy examination of Rosenfeld-stained cytopsin preparations. The cells were harvested from four individual guinea-pigs and added to separate plates (2×10^6 cells/well, in a 24-well polypropylene plate from Costar, Cambridge, MA; cell viability 90–95%, as determined by Trypan blue) and incubated for 2 h at 37°C, at 5% CO₂, in RPMI 1640 culture medium supplemented with 50 U/ml penicillin, 50 μ g/ml streptomycin and 2 mmol/ml 2-mer-

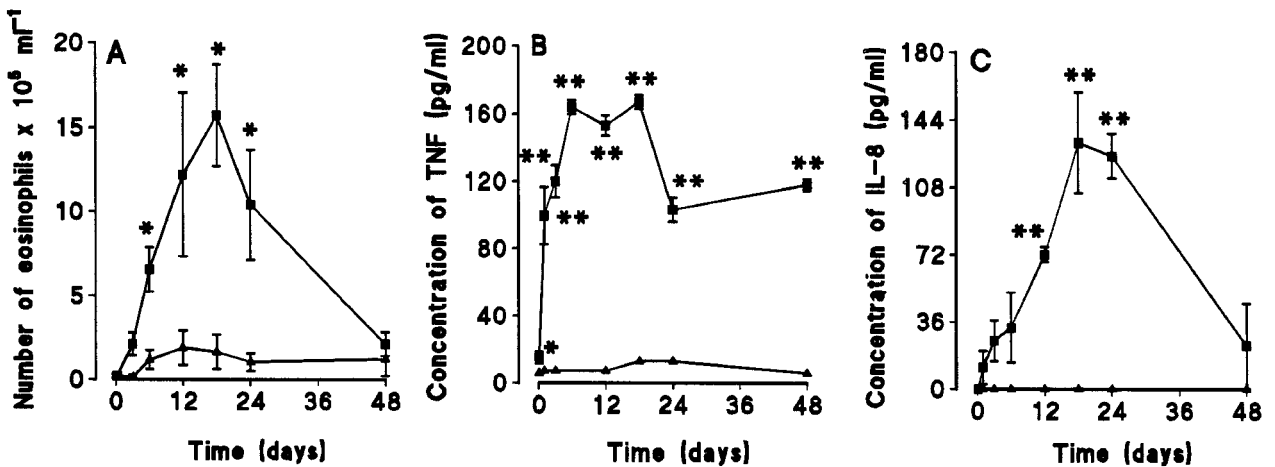


FIG. 1. Blood eosinophil counts (A) and serum TNF- α (B), and IL-8 (C) levels in *T. canis*-infected guinea-pigs sacrificed at different times post-infection ($n = 4-5$ per day). Asterisks indicate a significant difference between infected (■) and non-infected animals (▲). * $P < 0.05$ and ** $P < 0.01$.

captoethanol. The adherent cells were washed three times with medium to remove non-adherent cells. The monolayers of adherent cells were pre-incubated with RPMI 1640 medium supplemented with antibiotics and 10% FCS with or without anti-IL-5 Ab (0.17 ng/ml or 17 ng/ml) over a period of 15 min. Thereafter, medium or rIL-5 (0.5 ng/ml or 10 ng/ml) was added to the culture, and after 15 min of incubation the cells were stimulated or not with LPS (500 ng/ml). The plates were incubated for an additional 6–8 h at 37°C, 5% CO₂. The cell-free supernatants were collected, separated and kept at –20°C until the measurement of IL-8. The results were expressed as cytokine concentration (pg/ml) in supernatants of LPS-stimulated cells submitted to different treatments minus the concentration of cytokine released by cells incubated with medium alone. All the experiments were performed under aseptic conditions.

Statistical analysis

Data are presented as the mean \pm SEM and were analysed statistically by the Student's two-tailed *t*-test for differences between infected and non-infected animals, or between infected untreated and treated guinea-pigs. A $P < 0.05$ value was considered to be statistically significant. *In vitro* results were analysed statistically by the Student's two-tailed *t*-test and by ANOVA test.

Results

Blood eosinophil counts and serum TNF- α and IL-8 levels during *T. canis* infection

As shown in Fig. 1, *T. canis*-infected guinea-pigs developed blood eosinophilia (Fig. 1A) and had increased circulating TNF- α (Fig. 1B) and IL-8 (Fig. 1C)

levels. Blood eosinophilia increased significantly on day 12 and peaked by day 18 post-infection. Serum TNF- α levels increased 4 h after egg inoculation and became significantly higher 24 h later were still increased by day 3, peaked between days 6 and 18 and remained above control levels up to 48 days post-infection. TNF- α in the serum of non-infected animals was 8 ± 1 pg/ml, $n = 9$. Serum IL-8 concentration showed the same profile as blood eosinophilia, but increased significantly only on day 12 post-infection, reaching a peak between days 18 and 24 and decreasing by day 48. In this set of experiments, no IL-8 was detectable in the serum of non-infected guinea-pigs ($n = 7$).

BALF and femur flushing liquid IL-8 levels

BALF and bone marrow IL-8 levels were also determined during *T. canis* infection, and the results are shown in Fig. 2. BALF IL-8 levels were not detectable on day 3 but increased significantly on day 6 post-infection (41.3 ± 14.5 pg/ml), and reached a plateau between days 12 and 24 (72.2 ± 8.4 pg/ml on day 12, 72.5 ± 21.8 pg/ml on day 18; and 64.1 ± 10.9 pg/ml on day 24) post-infection. BALF IL-8 in non-infected guinea-pigs and femur flushing liquid IL-8 in *T. canis* infected guinea-pigs were not detectable at any of the times studied.

Effects of *in vivo* anti-IL-5 Ab treatment on IL-8 secretion

Having demonstrated that eosinophilia in *T. canis*-infected guinea-pigs is correlated with IL-5,³ and IL-8 secretion, we investigated the effect of IL-5 inhibition on TNF- α and IL-8 release into the serum of infected guinea-pigs. The i.p. injections of anti-IL-5 Ab (TRFK-5) into *T. canis*-infected guinea-pigs using the PrA (single

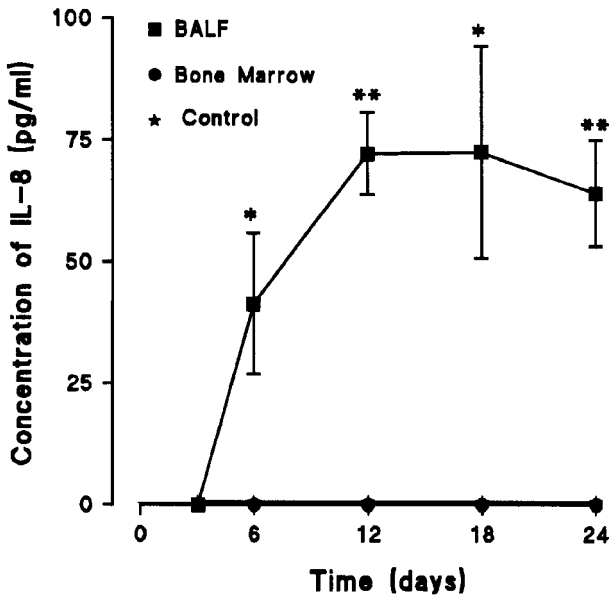


FIG. 2. BALF and bone marrow IL-8 levels in *T. canis* infected guinea-pigs sacrificed on different days post-infection ($n = 3$ per day). Asterisks indicate a significant difference between infected (■) and non-infected (★) animals. * $P < 0.05$ and ** $P < 0.01$.

dose) and PrB (several doses) protocols inhibited blood eosinophil counts by 97% to 99% as shown in Table 1. When the animals were treated with a single dose of anti-IL-5 Ab, PrA, a 159% increase in IL-8 was observed in serum (Table 1, range 101–234% of control; $n = 6$ animals, obtained in two different sets of experiments). Moreover, when infected animals were treated with PrB, eosinophilia was also suppressed and serum IL-8 levels were increased by 216% (Table 1, range 128–329% of control, $n = 6$, obtained in two sets of experiments). By contrast, no alteration in serum TNF- α concentration was observed whatever the treatment (Table 1, *T. canis*-infection without treatment $n = 7$, obtained from two set of experiments; anti-IL-5 Ab given once $n = 3$; several anti-IL-5 Ab injections $n = 5$). Infected animals treated with irrelevant Ab presented nonsignificant differences in serum IL-8 ($n = 3$) and TNF- α levels ($n = 3$). Serum IL-8 and TNF- α levels of non-infected animals were 14 ± 10

Table 1. Blood eosinophil counts and serum TNF and IL-8 levels in *T. canis* infected guinea-pig untreated or treated with TRFK-5

Treatment	Eosinophil counts	TNF (pg/ml)	IL-8 (pg/ml)
Untreated ^a	$12.2 \pm 5.3\blacklozenge$	$116.7 \pm 12.0\blacklozenge\blacklozenge$	$106.0 \pm 14.0\blacklozenge\blacklozenge$
Irrelevant Ab ^a	13.3 ± 4.5	85.5 ± 8.5	121.0 ± 9.0
TRFK-5 (PrA) ^a	$0.2 \pm 0.2^*$	118.5 ± 10.0	$274.4 \pm 42.0^{**}$
TRFK-5 (PrB) ^a	$0.01 \pm 0.01^*$	134.0 ± 12.0	$336.0 \pm 45.0^{***}$
Non infected	0.96 ± 0.41	8.0 ± 1.0	28.0 ± 14.0

^aThe animals were sacrificed 18 days after administration of 500 *T. canis* eggs by gastric intubation. In eosinophil counts the values represent mean \pm SEM $\times 10^5$ /ml. Diamonds indicate a significant difference between untreated infected animals and non-infected controls. * $P < 0.01$ and ** $P < 0.01$. Asterisks indicate a significant difference between untreated infected guinea-pigs and infected guinea pigs treated once (PrA) or five times (PrB) with anti-IL-5 Ab. * $P < 0.05$ and ** $P < 0.01$ and *** $P < 0.001$.

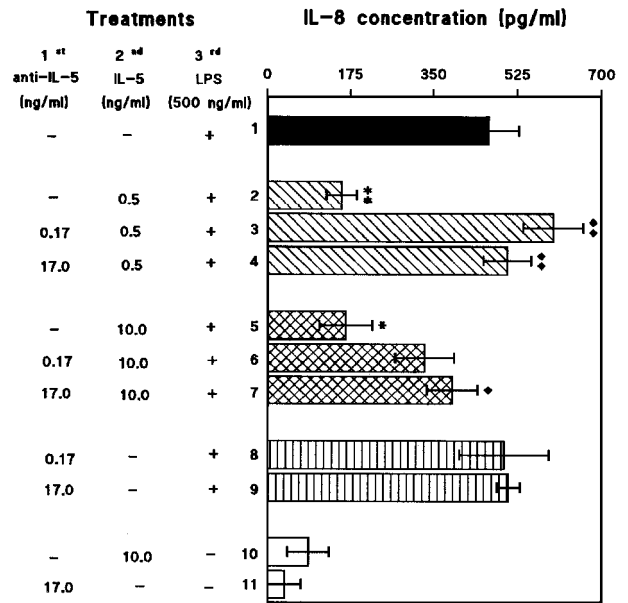


FIG. 3. Inhibition of IL-8 release into supernatants of LPS-stimulated guinea-pig adherent peritoneal cells pre-treatment with rIL-5. Data are as means \pm SEM ($n = 4$). Asterisks indicate differences between the amount of IL-8 released by LPS-stimulated adherent peritoneal cells pre-incubated with or without rIL-5, * $P < 0.05$ and ** $P < 0.01$. Diamonds indicate differences in the amount of IL-8 released by LPS-stimulated adherent peritoneal cells pre-treated with rIL-5 in the absence or presence of anti-IL-5 Ab, $\blacklozenge P < 0.05$ and $\blacklozenge\blacklozenge P < 0.01$.

and 8 ± 1 pg/ml, respectively; $n = 6$, obtained from two sets of experiments.

IL-5 downregulates IL-8 secretion *in vitro*

In order to clarify the *in vivo* experiments, *in vitro* experiments were performed using adherent peritoneal cells stimulated with LPS, a potent inducer of IL-8 secretion.¹² IL-8 was determined in supernatants of LPS-stimulated guinea-pig adherent peritoneal cells pre-incubated with or without rIL-5. The amounts of IL-8 released by adherent peritoneal cells stimulated with LPS (500 ng/ml) were increased in the absence of rIL-5 (Fig. 3, column 1). In the presence of rIL-5 (0.5 ng/ml or 10 ng/ml; Fig. 3, columns 2 and 5) LPS-

induced IL-8 release was inhibited by 64% and 66% respectively. Pre-incubation of adherent cells with two concentration of anti-IL-5 Ab (0.17 ng/ml or 17 ng/ml; Fig. 3, columns 3, 4, 6 and 7) prevented the IL-8 inhibition induced by 10 ng/ml of rIL-5 (Fig. 3, columns 6 and 7) or 0.5 ng/ml (Fig. 3, columns 3 and 4). A non-significant increase in IL-8 release followed the addition of anti-IL-5 Ab (0.17 ng/ml or 17.0 ng/ml; Fig. 3, columns 8 and 9) to LPS-stimulated cells. However, the addition of rIL-5 or anti-IL-5 Ab to cells without LPS failed to induce IL-8 release (Fig. 3, columns 10 and 11). Data are from $n = 4$ animals.

Discussion

This study demonstrated that the inoculation of guinea-pigs with *T. canis* induces a sustained increase of TNF- α levels in blood, and that the concentration of blood and BALF IL-8 increases later during infection, together with the number of circulating eosinophils. We demonstrated previously that *T. canis*-infected guinea-pigs present increased serum IL-5 levels followed by an increase in eosinophil numbers in blood and tissues which was suppressed by an anti-IL-5 Ab.³

TNF- α levels increased very early in the serum of infected guinea-pigs, reaching levels 87% above those of noninfected animals 4 h after egg inoculation and remaining above controls up to 48 days post-infection. *T. canis* larvae persist in different organs for long periods of time in infected animals²⁵ and release excretory-secretory antigens during migration as demonstrated before.²⁶ The prolonged and sustained TNF- α activity in blood may thus be explained either by the persistent presence of larvae in the tissues and/or by the release of those excretory-secretory antigens. Moreover, although TNF- α has been described as an inflammatory cytokine that appears first in the circulation in experimental sepsis and endotoxaemia and decreases a few hours after the stimulus,^{27,28} in chronic inflammation, as observed in mice injected with BCG,²⁹ *Paracoccidioides brasiliensis*,¹⁹ or *Schistosoma mansoni* eggs,³⁰ TNF- α is present for a long time post-infection. Moreover, in patients with septic shock due to various Gram-negative bacteria, serum TNF- α remained elevated in nonsurvivors who were still alive at day 10 after hospitalization.³¹ Thus, the increase of TNF- α levels for a long period of time is not limited to the present experimental model.

TNF- α is involved in eosinophil recruitment^{21,22} and has also been reported to occur in diseases presenting elevated numbers of eosinophils, such as airway inflammation.²² Finally, bronchoalveolar leukocytes from patients with bronchial asthma secrete high levels of TNF- α .³² The observation that serum and BALF IL-8 levels started to increase only after serum TNF- α release suggests that TNF- α may be

involved in the induction of IL-8 release in this model. Indeed, several reports have shown that TNF- α is a potent IL-8 inducer^{23,33} and IL-8 is present after TNF- α therapy in patients with chronic hepatitis.²⁴

The serum and BALF levels of IL-8 in infected animals increased significantly only between 6 and 12 days post-infection and peaked between days 18 and 24. These increases occurred at the same time as the increase in circulating eosinophil numbers, and the decrease of IL-8 in serum was followed by a reduction in blood eosinophil counts. Although blood eosinophilia in *T. canis*-infected animals was suppressed by anti-IL-5 Ab treatment, even when the anti-IL-5 treatment was performed 1 day before the peak of eosinophilia,³ we may suppose that IL-8 contributed to eosinophil recruitment. IL-8 has been described as an eosinophil chemoattractant.^{13,14} However, it appears that IL-8 is only able to induce eosinophil migration after this cell being primed by IL-5.^{14,34} Also, our results showing that IL-8 release occurs only outside the bone marrow (Fig. 2), suggest that this cytokine probably works as an eosinophil chemoattractant and contributes for the recruitment of IL-5-primed eosinophils from bone marrow to blood. Based on these data and on the data presented here, we suggest that IL-8 requires pre-priming with IL-5 to induce eosinophil recruitment *in vivo*. Thus, despite the release of high amounts of IL-8 in this model, the presence of IL-5 is essential for eosinophil migration, as demonstrated in antigen-challenged guinea-pigs⁶ and *in vitro*.^{14,34} However, the exact mechanisms involved in this process are not yet completely understood, and further studies using anti-IL-8 Ab will be essential to determine the contribution of IL-8 to the eosinophil recruitment and lung inflammation occurring in this model.³

Although TNF- α and IL-8 activities were measured in serum, BALF and supernatants of adherent peritoneal cells from guinea-pigs using a human ELISA kit, we think that the measurements refer to authentic guinea-pig cytokines presenting TNF- α and IL-8-like activities. TNF- α is highly conserved among species³⁵ and supernatants of guinea-pigs adherent peritoneal cells stimulated with LPS killed $45 \pm 6\%$ of WEHI cells in a cytotoxic assay, while supernatants from cells with only medium killed $4 \pm 4\%$ of cells ($n = 4$; data not shown); recombinant human IL-8 induces eosinophil migration when injected into the skin of guinea-pigs,¹³ IL-8 and TNF- α activities were also observed in the supernatants of guinea-pig adherent peritoneal cells after LPS stimulation, as observed in human cells.^{12,36,37} Moreover, eosinophil accumulation in guinea-pig skin induced by LPS was inhibited by anti-TNF- α receptor,²¹ and serum IL-8 and TNF- α levels were decreased in *T. canis*-infected guinea-pigs treated with dexamethasone (data not shown).

To better characterize the IL-5/IL-8 interactions we investigated the changes in serum TNF- α and IL-8

levels in *T. canis*-infected guinea-pigs after IL-5 depletion by an anti-IL-5 Ab. When IL-5 was thus suppressed in infected animals eosinophil counts were also reduced and serum IL-8 levels increased, in the absence of changes in serum TNF- α (Table 1). These results suggest that IL-5 downregulates the expression of IL-8. These *in vivo* data were confirmed by *in vitro* experiments: IL-8 production in the supernatants of LPS-treated adherent peritoneal cells in the presence or not of rIL-5 (Fig. 3). IL-8 release (Fig. 3) were increased by LPS, as reported.³⁷ However, when rIL-5 was added to cells before LPS, a marked inhibition of IL-8 release occurred, which was prevented by anti-IL-5 Ab treatment, demonstrating specificity. Also, these results were corroborated by the findings that rIL-5 added to LPS stimulated adherent peritoneal cell culture induced significant inhibition of mRNA IL-8 expression which was reversed by the addition of anti-IL-5 Ab (data not shown).

Thus, the inhibitory effects of IL-5 on IL-8 secretion may represent an endogenous mechanism of down-regulating eosinophil inflammation. It may be possible that IL-5 is required to prevent IL-8 over-production and release. Moreover, as discussed above, we suggest that serum IL-8 acts as an eosinophil chemoattractant in the presence of IL-5. However, since IL-5 is decreased during the course of infection an increase in serum IL-8 occurs which, above a certain level, may act as a modulator of eosinophil release from bone marrow and tissue eosinophil migration. Thus, it is possible that the increase of serum IL-8 levels in *T. canis*-infected guinea-pigs after anti-IL-5 Ab treatment contributes to the inhibition of eosinophilia. IL-8, which is chemotactic for neutrophils, inhibited the migration of these cells to the skin when administered at high concentrations by the iv. route.³⁸ On the other hand, eosinophils have been described as tissue-resident cells that remain only a few hours in the circulation,³⁹ and this characteristic may explain why we did not find an increase in blood eosinophil counts after anti-IL-5 Ab treatment.

The present results, describing a new function of IL-5 as a modulator of IL-8 secretion, may be useful for a better understanding and control of diseases presenting eosinophilia and high IgE levels.¹ IL-8 selectively inhibits IgE production⁴⁰ and may be involved in the control of serum IgE levels *in vivo*. Production of high amounts of IL-5 in such diseases may downregulate IL-8 levels and consequently contribute to the maintenance of high IgE levels during infection. Thus, an anti-IL-5 Ab treatment might be useful both to inhibit eosinophil counts and to increase serum IL-8 production which down-regulates IgE secretion.

In summary, the results presented here demonstrate that in *T. canis*-induced eosinophilia there is a sequential release of TNF- α and IL-8 occurring in parallel to or after serum IL-5 release. In this model,

IL-5 appears to be the main factor involved both in the induction of eosinophilia and in the *in vivo* control of IL-8 synthesis and production. Thus, IL-5 may also contribute to the increase of IgE synthesis observed in parasitic diseases and allergy, in parallel to eosinophilia.

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