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Trichinella spiralis infection rapidly induces lung inflammatory response The lung as the site of helminthocytotoxic activity

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ABSTRACT

In the present work, we studied the kinetics of the appearance of different immunological parameters in the lungs during the intestinal phase of infection with *Trichinella spiralis*. We also evaluated the lung's role in the retention and death of this helminth in its migratory stage. To study these parameters, we used lung extracts, lung cell suspensions and rat lung tissue sections.

During the intestinal phase of infection (days 0–13 post-infection, p.i.), an inflammatory response is elicited in the lungs, which reflects humoral, cellular and functional changes. These changes included an increased number of mast cells and eosinophils and the local production of IL-4, IL-5, IL-10, TNFα, IFNγ, IL-13, CCL11 and CCL28. We found hyperplasia of the bronchus-associated lymphoid tissue (BALT). Total and specific IgA, IgE, IgG1 and IgG2a were detected locally. The retention of the migratory larvae in the lung, together with the *ex vivo* cytotoxic capacity of the lung cells and antibodies present in the lung extracts, suggested that the lung was one of the immune defense organs against the pathogen's migration stage.

Introduction

Different helminth species possess an obligatory route through the lungs and induce an alteration of the immunological status (Silveira et al. 2002; Reece et al. 2006). The relationship between the appearance of such processes and their influence on the course of infection has not been described. Lung manifestations during these phases are not common, suggesting tolerance by the host.

During the systemic migration toward the striated muscles, a number of migrant newborn larvae (NBL) of *Trichinella spiralis* migrate through different organs (Despommier et al. 2005), i.e., the lungs. Harley and Gallicchio (1971) studied the migration of *T. spiralis* in orally infected rats and isolated the NBL from the lungs. However, they did not determine whether the parasites were only passing through or were retained in such organs. According to the literature, the lungs would act as a parasite entrapment and destruction site (Binaghi et al. 1981; Wang and Bell 1986; Bruschi et al. 1992). These findings suggest that the lungs provide a suitable environment for retention and defense processes.

The effector immune mechanism against NBL is related to the presence of antibodies (Abs) that have specificity against the NBL surface antigen (Ag) and leukocytes that function as effector cells through antibody-dependent cell-mediated cytotoxicity (ADCC) reactions (Kazura 1981; Gansmüller et al. 1987; Venturiello et al. 1993, 1995). Nevertheless, the precise location in the host's body where such mechanisms occur remains unknown. Because the size of the NBL is on the same order of magnitude as lung blood capillaries, NBL retention may occur in this organ, where the parasite might be attacked by effector cells and specific Abs. Only the larvae capable of evading this mechanism would invade the striated muscle.

In our previous work, we demonstrated that on its migratory route through the lung, *T. spiralis* NBL evoke an inflammatory allergic response together with bronchus-associated lymphoid tissue (BALT) and goblet cells hyperplasia (Venturiello et al. 2007). The parameters of the development of this inflammatory process remain to be elucidated.

The mucosal immune system is an integrated system, and the immunization at one site may produce a protective immunity at another site (Lamm and Phillips-Quagliata 2002; Roux et al. 2003;

Abbreviations: BALT, bronchus-associated lymphoid tissue; NBL, newborn larvae; ADCC, antibody-dependent cell-mediated cytotoxicity; Abs, antibodies; Ag, antigen; Igs, immunoglobulins; ML, muscle larvae; p.i., post-infection; LTEs, lung tissue extracts; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ML-ESP, muscle larvae excretory–secretory products; Ig LSCs, immunoglobulins lung secreting cells; PR, positivity ratios.

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Holmgren and Czerkinsky 2005). Our results suggested that the inflammatory process observed during *T. spiralis* infection in the lung results from a signal that originated in the gut due to the penetration of the muscle larvae (ML) into the gut epithelium, which develops an inflammatory process that ends with the rejection of the adult worms from such mucosa (Ierna et al. 2008).

Taking into account this evidence and previous findings, we aimed to characterize the kinetics of the appearance of the immunological parameters involved in the development of the inflammatory process in the lung during the intestinal phase of *T. spiralis* infection in rats and to evaluate the role of the lung in the retention and death of the parasite.

Materials and methods

Animals and infection

Two-month-old female Wistar rats were orally infected through a gastric canula with 2000 ML per rat. The ML were obtained from the muscle tissue of Swiss mice by the artificial digestion method (Nuñez et al. 2005). As controls, uninfected animals were administered PBS orally by gastric canula. A minimum of five animals per were used in each experiment. The animals were provided with water and food *ad libitum*, and they were exposed to 12 h light–dark cycles; the room temperature was kept at 21 ± 1 °C. All the experiments were approved by the Review Board of Ethics of the Instituto de Estudios de la Inmunidad Humoral (IDEHU) and conducted in accordance with the guidelines establish by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

Histological and immunohistochemical analysis of lung tissue

The lower respiratory tract was removed from the animals ($n = 5$–6/day p.i.) that were sacrificed on days 1, 2, 3, 6 and 13 p.i. and from the controls, and the removed tissue was subjected to the Sainte-Marie technique (Sainte-Marie 1961). Paraffin from the tissue sections was removed as previously described (Venturiello et al. 2007).

The Giemsa (Biopur, Rosario, Argentina) staining was employed to visualize the BALT, and Alcian Blue 8GX (CI 74240; Mallinckrodt, St. Louis, MO, USA)-PAS/Gill's Hematoxylin (Biopur) was used to assess the hyperplasia and the number of goblet cells. The goblet cell count was performed in the epithelium located close to the BALT and in the primary and secondary bronchial epithelium at 400× magnification. The results were expressed as the number of goblet cells/100 epithelial cells. The eosinophil and mast cell counts in the lung parenchyma were completed in sections stained with the modified Luna's methodology (Tomasi et al. 2008) and Toluidine blue (CI 50240; Merck, Darmstadt, Germany) (Tomasi et al. 2003), respectively. The eosinophils and mast cells were counted in 100–150 randomly selected fields for each sample at 400× magnification employing a grid with a known area (62,500 μm^2 at 400× magnification) attached to a microscope ocular (Olympus, Tokyo, Japan). The results were expressed as the number of cells/mm^2.

To determine the phenotype of the cells present in the BALT, the lung tissue sections were incubated with the following antisera: (1) IgE^+ cells: a goat anti-rat IgE serum (Bethyl Laboratories, Inc., Montgomery, TX, USA), followed by a FITC-conjugated anti-goat IgG serum (Sigma, St. Louis, MO, USA); (2) $CD4^+$ cells: a mouse anti-rat CD4 mAb (Pharmingen, San Diego, USA), followed by a FITC-conjugated $F(ab')_2$ anti-mouse IgG serum (ICN, Cappel, Aurora, OH, USA); and (3) IgA^+ cells: a FITC-conjugated anti-rat IgA serum (Bethyl Laboratories). The number of cells in the BALT was recorded for 15 fields at 1000× magnification using a fluorescence microscope (Olympus, Tokyo, Japan) by two independent observers.

Obtention of lung tissue extracts (LTEs)

The LTEs were obtained on days 1, 2, 3, 6 and 13 p.i. using the Perfext method (Villavedra et al. 1997) with slight modifications to detect total and specific immunoglobulins (Igs), cytokines and chemokines. Briefly, the rats ($n = 5$/day p.i.) were bled and infused with PBS plus heparin (5000 UI/ml) into the heart. The perfused organs were cut into small pieces, placed in an extraction solution containing 90 mM CHAPS (Research Organics, Cleveland, USA) in PBS and protease inhibitors (EDTA-free Complete, Roche Diagnostics, Mannheim, Germany) at 2 μl/mg of tissue and frozen at −70 °C. After thawing, the extraction was performed overnight at 4 °C using a homogenizer. After centrifugation, the supernatants were collected, filtered through a 0.22 μm filter (Millipore Co, Bedford, MA, USA), aliquoted and kept frozen at −70 °C until use.

Lung cell suspensions

The perfused lungs were obtained as described above and removed. The cell suspensions were prepared by cutting the tissue into small pieces and later digested in RPMI (Gibco, Grand Island, NY, USA) plus collagenase A (0.5 mg/ml, Roche Diagnostics), DNase (0.1 mg/ml, Roche Diagnostics), L-glutamine (1.46 g/100 ml, Gibco), penicillin (100 IU/ml) and streptomycin (100 μg/ml, Gibco) for 40 min at 37 °C with occasional shaking. The tissues were homogenized, and the cell suspensions were passed through a 40-μm nylon sieve. The remaining erythrocytes were lysed with isotonic ammonium chloride buffer. The cell suspensions were resuspended and washed twice with PBS plus 5 mM EDTA (Gibco) and 3% fetal calf serum (FCS, Gibco), and the cells were counted using a hemocytometer and Trypan blue dye (bioMérieux, Marcy L'Etoile, France). The cell viability was invariably higher than 95%.

Finally, the cell suspensions were suitably resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 100 μg/ml streptomycin, 100 IU/ml penicillin (Gibco) and 5% FCS (Gibco).

Detection of NBL in the lungs

To detect the NBL in tissue sections, we used histochemical techniques and immunofluorescence using a reference human anti-NBL surface serum followed by a FITC-conjugated anti-human serum (Sigma). The *T. spiralis* in the stage of NBL was also recovered from the lung cell suspensions ($n = 5$/studied day) that were obtained as described above and subsequently counted in grooved Petri dishes and observed using a microscope by two independent observers. To assure that the enzymatic treatment did not kill the larvae, the normal lungs spiked with viable NBL were also processed as positive controls. Because the NBL are shed into the bloodstream and lymphatic circulation from the gut by the adult worms on and after day 5 p.i. (Denham and Martínez 1970; Despommier et al. 2005), these methodologies were conducted on days 6 and 13 p.i.

ELISPOT assay

To detect the presence of the total and specific anti-muscle larvae excretory–secretory products (ML-ESP) IgA, IgE, IgG1 and IgG2a lung secreting cells (Ig LSCs), ELISPOT assays were performed on days 3, 6 and 13 p.i. as previously described (Czerkinsky et al. 1983) with slight modifications. Briefly, 96-well nitrocellulose-bottom microtiter MultiScreen HTS plates (Millipore) were coated overnight at 4 °C with ML-ESP (50 μg/ml) obtained as described by Nuñez et al. (2000) or the following capture antisera: goat anti-rat IgA serum (Bethyl Laboratories); sheep anti-rat IgE serum (Bethyl Laboratories); and goat anti-γ1 or goat anti-γ2a chain serum (Bethyl Laboratories). After washing in sterile PBS, the plates

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were blocked with 10% FCS in DMEM for 1 h at 37 °C. Then, different numbers of lung cells/well from infected or control rats were added and incubated for 24 h at 37 °C in a moist atmosphere containing 5% CO_2. The wells were then washed in PBS and PBS with 0.05% Tween (PBST) and incubated with HRP-conjugated anti-rat IgA, IgG1 or IgG2a serum (Bethyl Laboratories) suitably diluted in 0.05% PBST at room temperature. For the detection of specific and total IgE, biotinylated mouse anti-rat IgE serum (BD Biosciences, San Diego, CA, USA) was added and incubated for 1 h at room temperature, followed by the addition of a macromolecular complex of avidin and biotinylated peroxidase (Vector, Burlingame, CA, USA). The spots were developed with a 3-amino-9-ethylcarbazole-H_2O_2 (Sigma) solution incubated at room temperature. The reaction was stopped by adding tap water. After the plates dried, the spots were enumerated using an Immunospot reader (CTL, Cleveland, USA). The reactions were performed in triplicate with three groups of rats and pooled cell suspensions ($n = 5$/day p.i.). The results were expressed as the number of Ig LSCs per 10^6 leukocytes.

Detection of total and specific Igs in LTEs

Total IgA, IgE, IgG1 and IgG2a were determined by a capture ELISA commercial kit (Bethyl Laboratories). Levels of specific anti-ML-ESP IgA, IgE, IgG1 and IgG2a were determined by indirect ELISA. Briefly, flat-bottomed PolySorp polyvinyl microtiter plates (Nunc, Roskilde, Denmark) were coated with ML-ESP (5 μg/ml) that was obtained as described by Nuñez et al. (2000). After overnight incubation at 4 °C, the plates were washed in 0.05% PBST and then blocked using a 5% BSA solution for 1 h at 37 °C. After washing, the LTEs from control animals and those obtained on days 1, 2, 3, 6, and 13 p.i. were added and incubated for 1 h at 37 °C. Plates were washed and incubated again in the same conditions with the corresponding anti-Ig sera (Bethyl Laboratories) suitably diluted in 0.05% PBST and incubated for 1 h at 37 °C. The reactions were developed with a tetramethylbencidine/H_2O_2 solution. The color reactions were read at dual wavelengths 450 and 600 nm. The total Ig concentrations were obtained from the standard curves. The specific Ig levels were expressed as positivity ratios (PR) = OD of infected animals' LTEs/OD of control animals' LTEs. The samples with a PR > 1 were considered positive. All of the samples were assayed in duplicate.

Anti-NBL surface IgE, IgA, IgG1 and IgG2a were detected using the indirect immunofluorescence assay on slides containing methanol-fixed NBL obtained by the method previously described (Nuñez et al. 2002). The slides were incubated overnight at 4 °C with serially diluted LTEs from the control animals and those obtained on days 6 and 13 p.i. Then goat anti-rat IgE serum, goat anti-rat IgA serum, goat anti-rat IgG1 serum or goat anti-rat IgG2a serum (Bethyl Laboratories) suitably diluted in 0.1% PBST was added and incubated for 1 h at 37 °C. After washing, the FITC-conjugated anti-goat IgG serum (Sigma) suitably diluted in 0.1% PBST plus Evans blue was added, and the slides were then incubated for 1 h at 37 °C. The slides were air-dried and mounted in buffered glycerol. The LTE samples were considered positive when the fluorescence of the parasite surface was observed using a fluorescence microscope (Olympus). The reference anti-NBL surface rat serum and control rat serum were employed as the positive and negative controls, respectively. All of the reactions were performed in duplicate using LTEs obtained from two different groups of rats ($n = 5$ at each day p.i. studied).

Detection of cytokines and chemokines in LTEs

Commercial ELISA kits were used to determine the levels of INFγ, TNFα, IL-10, IL-4 (BD Biosciences) and CCL11 (eotaxin; R&D Systems, MN, USA) in the LTEs obtained from animals infected on days 1, 2, 3, 6 and 13 and the controls. The levels were derived from standard curves and expressed as pg/ml.

The levels of IL-5, IL-12, IL-13 and CCL28 (MEC) were determined by an immunoelectrotransfer blot assay with the following antisera: a mouse anti-rat IL-5 (R&D Systems), a mouse anti-rat IL-12 (BioSource International, Inc., Camarillo, CA, USA), a mouse anti-rat IL-13 (BioSource International) and a goat anti-CCL28 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). A biotinylated horse anti-mouse (Vector) and HRP-conjugated donkey anti-goat antisera (Santa Cruz Biotechnology) were used as the detection reagents. When appropriate, the reaction was followed by a macromolecular complex of avidin and biotinylated HRP (Vector). All of the reactions were developed using a solution of α-chloronaphtol/H_2O_2. The reactions were stopped by the addition of tap water. To eliminate any variability in the results due to the amount of protein seeded in each gel lane, a goat anti-β-actin serum was used in parallel (Santa Cruz Biotechnology). The membranes were analyzed by densitometry using the Scion Image Pro software (Bethesda, MD, USA), and the cytokine and CCL28 levels were expressed as PR according to the following formula: PR = [ODc/ODβ] infected/average [ODc/ODβ] control animals, where ODc is the optical density corresponding to the cytokine or CCL28 band, and ODβ is the optical density corresponding to the β-actin band. The reactions were considered positive when the PR > 1. All of the reactions were performed in duplicate using LTEs obtained from two different groups of rats ($n = 5$ at each day p.i. studied).

Ex vivo ADCC assay

To assess the helminthocytotoxic activity of lung cells and LTE samples, an ADCC assay was performed in flat-bottomed microwell modules (Nunc) as previously described (Venturiello et al. 1993). Preliminary studies were performed to determine if the lung cells obtained from the control ($n = 6$) and infected animals ($n = 6$) on day 6 p.i. had helminthocytotoxic activity when exposed to the reference anti-NBL surface cytotoxic rat serum capable of killing the NBL in the presence of cells from other tissues (Venturiello et al. 1995). Briefly, a suspension containing 50 NBL obtained by the method previously described (Nuñez et al. 2002) was placed in each well, and the exact number of the larvae was counted. We added 20 μl of undiluted reference anti-NBL surface cytotoxic rat serum and 100 μl of lung cells suspension containing 5×10^6 leukocytes/ml to each well. The microwells were kept for 18 h at 37 °C in a moist atmosphere of 5% CO_2. The NBL death was measured using direct microscopy by two independent observers, and the mortality rate was calculated using the following formula: % Mortality = $[(NBL_{\text{initial number}} - NBL_{\text{final number}})/NBL_{\text{initial number}}] \times 100$. The unspecific mortality was determined using rat sera obtained from control rats, and the values obtained were subtracted from those obtained with infected animals. After corroborating this activity, the ADCC assay was performed as described previously using undiluted LTEs obtained on days 6 ($n = 2$) and 13 p.i. ($n = 8$) containing anti-NBL surface Abs. The results were calculated as previously described. The unspecific mortality was determined using the LTEs ($n = 8$) obtained from control rats. No cytotoxic activity against NBL was found when the NBL were incubated in the absence of the lung cells. The positive controls were performed using anti-NBL surface cytotoxic serum. All of the experiments ($n = 3$) were performed in duplicate.

Statistical analysis

The data were analyzed using the two-way ANOVA test, but when the study variables violate the normality assumptions (Shapiro–Wilks test) and the homogeneity of variance (Levene's test), the non-parametric Kruskal–Wallis ANOVA was used. The

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one-way ANOVA test was used for the statistical analysis of CCL28 levels. A P-value < 0.05 was considered significant. The dates were analyzed using the SPSS 16.0 statistical software. The specific test employed is indicated in each figure.

Results

Histological changes in lung tissue

The microscopic examination of the lung sections that were subjected to Giemsa stain found BALT hyperplasia on and after day 2 p.i., and the hyperplasia gradually increased during the course of the present study. Moreover, a marked allergic lung inflammation was observed throughout the study period. This response included the following characteristics: (1) the inflammation of the interalveolar and bronchoarterial spaces, (2) the presence of eosinophils and mast cells scattered homogeneously in the parenchyma, with the localization of eosinophils in the perivascular and peribronchial regions and (3) the pleural infiltration with eosinophils and mast cells (Fig. 1). It is important to highlight that the eosinophils and mast cells were also observed in the BALT.

The goblet cell hyperplasia was observed on and after day 1 p.i. and increased significantly ($P \ll 0.05$) on and after day 3 p.i. (9 ± 1 *vs.* 2 ± 0 goblets cells/100 total epithelial cells for the infected *vs.* control animals), with a peak value of 17 ± 1 and 18 ± 2 on days 6 and 13 p.i. (Fig. 2A). A large amount of mucins was also observed in the alveolar spaces. It is noteworthy that most of the goblet cells were observed in the epithelium close to the BALT. The number of eosinophils in the lung parenchyma was significantly elevated ($P \ll 0.05$) from on and after day 1 p.i. (49 ± 10 *vs.* 14 ± 2 eosinophils/mm^2 for the infected *vs.* control animals), reaching maximum levels on day 13 p.i. (171 ± 14 eosinophils/mm^2) (Fig. 2B). The mast cell counts significantly increased ($P = 0.004$) between days 1 and 6 p.i. (12.6 ± 1.8, 8.9 ± 1.5, 9.5 ± 1.5, 9.4 ± 0.6 *vs.* 4.3 ± 0.5 mast cells/mm^2), for infected *vs.* control animal (Fig. 2C).

Immunohistochemical studies showed that the IgE$^+$ cells residing in the BALT of infected animals were found to be elevated from day 1 p.i. in 50% of the infected animals and after day 2 p.i. in 100% of the animals. This increase was significant ($P = 0.0001$) after day 3 p.i. On day 1 p.i., we observed a significant decrease in the numbers of IgA$^+$ cells ($P = 0.0001$). The number of CD4$^+$ cells significantly increased ($P = 0.0001$) after day 3 p.i. (Table 1).

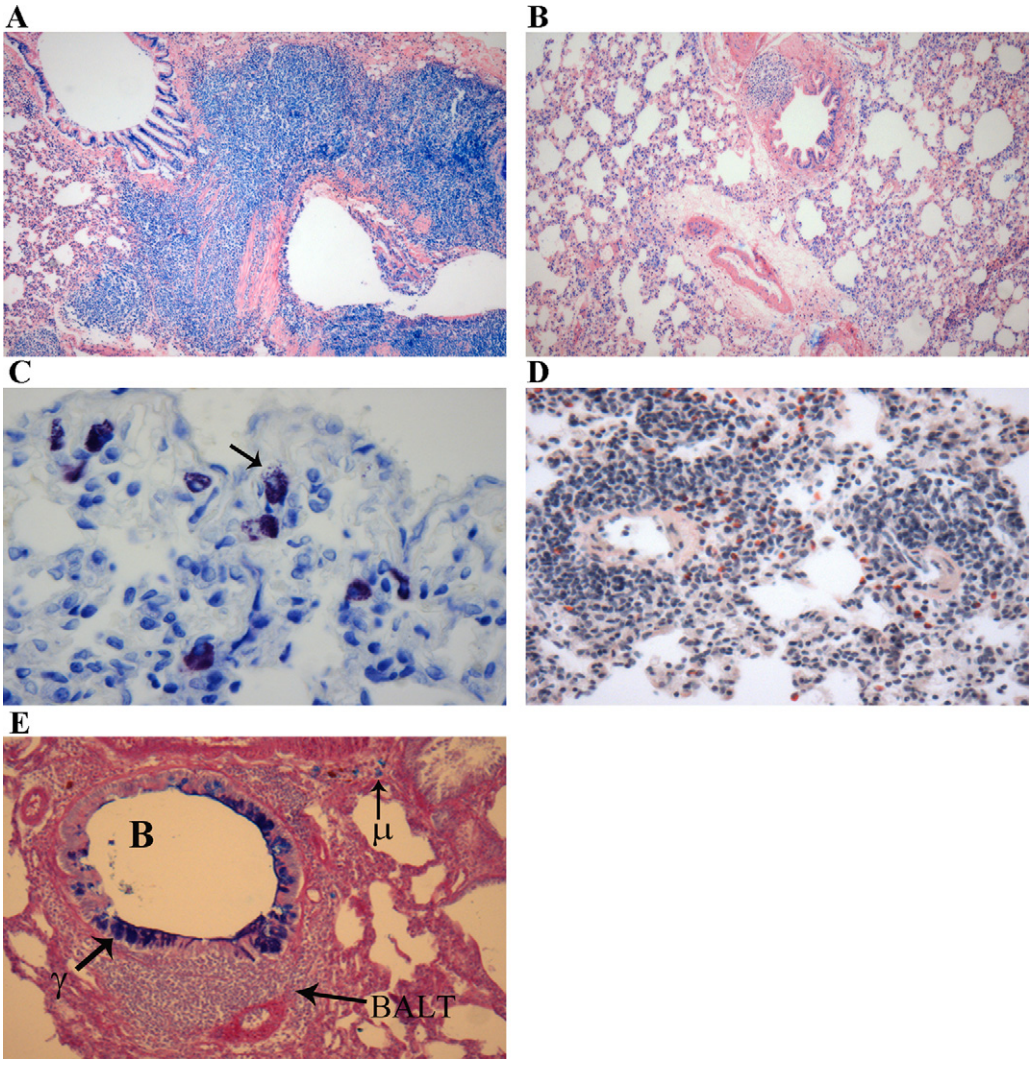

Fig. 1. Histopathology of the lung during the early phase of *T. spiralis* infection in rats. (A) Lung tissue section from a rat infected with *T. spiralis* on day 3 p.i. and the BALT hyperplasia and thickening of the alveolar septa by inflammatory infiltrates (40×, Giemsa). (B) BALT from control rats (arrow), with histologically conserved parenchyma (40×, Giemsa). (C) Mast cells infiltrate on day 3 p.i. (400×, Toluidine blue). The arrow indicates a degranulated mast cell. (D) Inflammatory infiltrate of eosinophils scattered in the lung parenchyma on day 2 p.i. (400×, Luna's methodology). (E) Goblet cell hyperplasia in the bronchial epithelium (γ) and mucus secretion on day 3 p.i. (400×, Alcian blue-PAS/Gill's hematoxylin). This figure shows mast cells (μ) and BALT. Five individual rats/group were examined.

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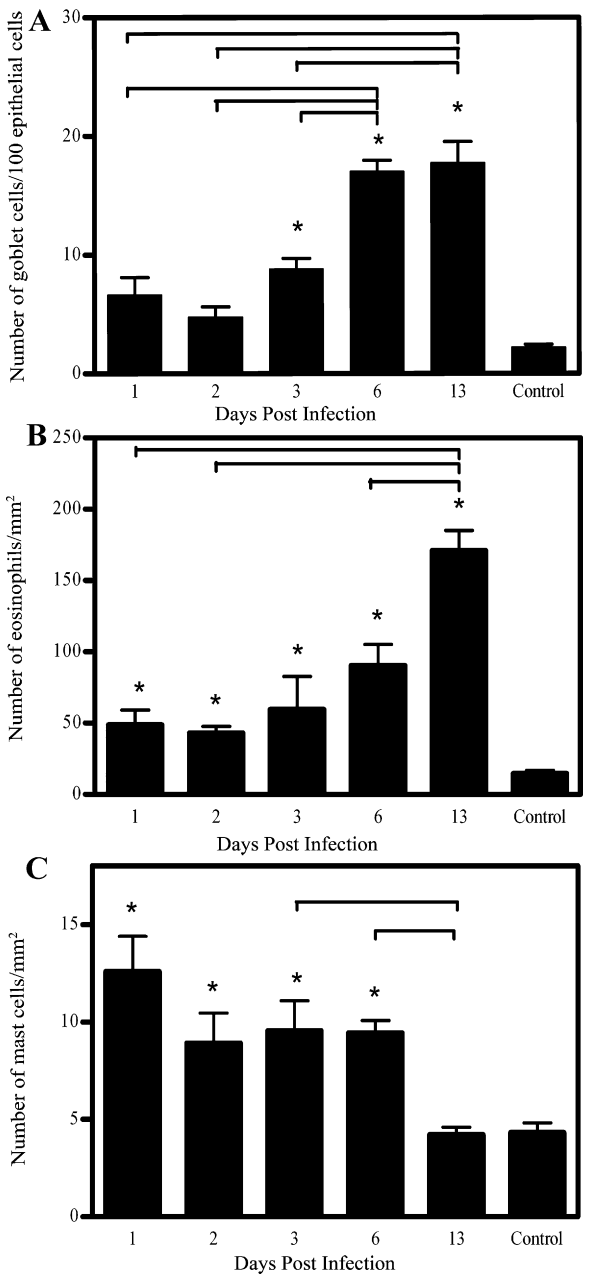

Fig. 2. Kinetics of the appearance of goblet cells, eosinophils, and mast cells in the lung during early *T. spiralis* infection. The cells were counted on different days p.i. in tissue sections at 400× magnification. (A) The goblet cells were counted in the lung epithelium (Alcian blue-PAS/Gill's hematoxylin staining). (B and C) The eosinophils and mast cells were counted in the lung parenchyma tissue sections using Lunas' methodology and Toluidine blue stains, respectively, in a grid with a known area (62,500 μm^2 at 400× magnification) attached to the ocular of the microscope. The data presented here are the mean numbers of cells ± SEM from 5 rats/day p.i. counted through light microscopy observation by two independent observers. The asterisks indicate significant differences between infected and control animals (goblet cells and eosinophils, $P \ll 0.05$, two-way ANOVA test; mast cells, $P = 0.004$, non-parametric Kruskal–Wallis ANOVA). The bars indicate significant differences between the indicated days.

Detection of NBL in the lungs

Dead NBL were recovered from the lung cell suspensions (210 ± 28 NBL on day 6 p.i. *vs.* 1 ± 1 NBL on day 13 p.i.) and detected in the tissue sections on days 6 and 13 p.i. These NBL were found in the lung parenchyma as well as in the alveoli (Fig. 3).

Table 1
Cell phenotype in the bronchus-associated lymphoid tissue (BALT) during the early phase of *Trichinella spiralis* infection in rats.

Days p.i.	No. IgA^+ cells	No. IgE^+ cells	No. $CD4^+$ cells
1	170 ± 18[b]	108 ± 19	137 ± 17
2	351 ± 53	135 ± 10	199 ± 29
3	402 ± 4	197 ± 19[d]	307 ± 32[h]
6	313 ± 19	267 ± 5[e]	528 ± 65[i]
13	256 ± 11	250 ± 15[f]	247 ± 11[j]
Control	263 ± 14[a]	88 ± 11[c]	195 ± 11[g]

Data are expressed as mean ± S.E.M. ($n = 5$/day p.i.) of number (No) cells/15 fields at a 1000× magnification. Significant differences between infected and control rats were observed in the BALT. $P = 0.0001$: b *vs.* a; d, e, f *vs.* c; h, i, j *vs.* g. Data were analyzed by a two-way ANOVA test.

Cytokine and chemokine levels

The levels of cytokines and chemokines were measured in the LTE during the early phase of *T. spiralis* infection and are depicted in Fig. 4. Significantly high levels of TNFα and IFNγ were present in the lungs shortly after the entrance of ML in the gut ($P \ll 0.05$ and $P = 0.0001$, respectively). The TNFα level was significantly elevated until day 2 p.i. (370 ± 54 *vs.* 140 ± 43 pg/ml for infected *vs.* control animals), while the IFNγ level was sustained until day 6 p.i. compared with the controls (233 ± 35 *vs.* 80 ± 7 pg/ml).

IL-4 and IL-13 are key cytokines in the induction and elicitation of Th2 responses, and their kinetics were demonstrated. We found that despite the elevation of the levels of IL-4 in 100% of the animals from day 1 p.i., these values increased significantly ($P = 0.003$) toward the end of the study period on days 6 and 13 p.i. (111 ± 25, 239 ± 86 *vs.* 11 ± 7 pg/ml), whereas the PR for IL-13 was elevated only until day 6 p.i. and returned to baseline levels on day 13 p.i. We found highly significant values ($P \ll 0.05$) on days 1 through 6 p.i. compared with the values observed on day 13 p.i. On the other hand, the PR for IL-5 was slightly elevated throughout the study period. The IL-10 levels were significantly higher ($P = 0.004$) on days 3 through 6 p.i. (infected animals: 142 ± 12, 124 ± 10 *vs.* control animals 68 ± 4 pg/ml). The rise in the levels of the latter cytokine was coincident with the fall in the levels of TNFα and the increase of IL-4. The PR for IL-12 was sustained, and we found no significant differences throughout the study period. To analyze the cell recruitment signals, we assessed the levels of CCL11 and CCL28. CCL11, a key chemokine involved in eosinophil trafficking (Dixon et al. 2006), was present at high levels ($P = 0.0002$) throughout the study period, and we found peak values on days 1 and 13 p.i. compared with control values (1809 ± 148, 1727 ± 285 *vs.* 286 ± 25 pg/ml, respectively). Nevertheless, a significant decrease was found for the latter chemokine between days 3 and 6 p.i. CCL28, another chemokine involved in the trafficking of immune cells (John et al. 2005), was found at high levels ($P = 0.001$) in 100% of the animals during the first two days p.i.

Production and secretion of total and specific Ig isotypes

Igs in LTE: Total and specific Ig levels in the LTEs are depicted in Fig. 5. The total IgA levels were found to be high from day 1 p.i. in 75% of the animals and significantly different ($P \ll 0.05$) from the control animals from day 6 p.i. (1361 ± 140 *vs.* 562 ± 85 μg/ml). The maximum levels of this Ig were found on day 13 p.i. (1628 ± 165 μg/ml). The latter values were 2.5 times higher than those of the control animals. The anti-ML-ESP IgA was readily detected from day 1 p.i. in 50% of the infected animals, reaching maximum levels on day 6 p.i. ($P = 0.002$).

Total IgE levels were found to be increased from day 1 p.i. in 100% of the animals compared with levels in control animals ($P \ll 0.05$) on days 6 and 13 p.i. The peak values of this Ig were found on day

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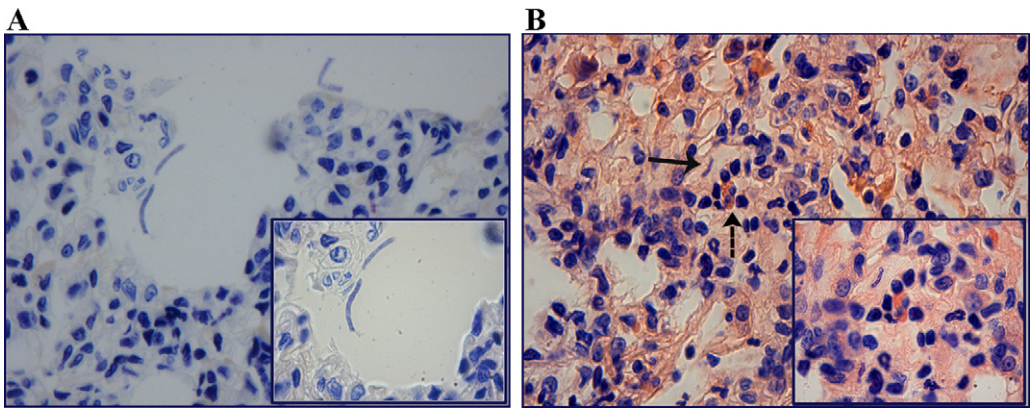

Fig. 3. Detection of *T. spiralis* NBL in lung sections. (A) Host cells attached to NBL surfaces in lung alveoli (400×, Toluidine blue). In the inset, the NBL are shown at high magnification (1000×). (B) NBL in the lung parenchyma surrounded by inflammatory cells; the shaded arrow indicates the NBL, and the dashed arrow indicates an eosinophil cell (400×, H&E). Inset magnification 1000×.

13 p.i. (5810 ± 1604 *vs.* 6.8 ± 3.44 ng/ml). Moreover, the level of Ig was 200 times higher than baseline levels. The anti-ML-ESP IgE was found in 100% of the infected animals from day 1 p.i. and the levels of this specific Ig were sustained throughout the study period, except on day 6 p.i., when the levels were significantly lower ($P = 0.05$), and only 33% of the animals were positive.

Total IgG1 levels increased ($P \ll 0.05$) on and after day 3 p.i., with peak values on day 13 p.i. (17.48 ± 0.33 *vs.* 1.90 ± 0.04 ng/ml). The latter Ig levels increased by 9.2 times compared with levels in the control animals. Anti-ML-ESP IgG1 was detected in 56% of the animals on day 3 p.i. and significantly decreased ($P = 0.001$) on day 6 p.i., whereas by day 13 p.i., all of the animals had significantly increased levels of this specific Ig ($P = 0.001$).

Total IgG2a levels significantly ($P \ll 0.05$) increased on and after day 3 p.i. (10.14 ± 0.50 *vs.* 2.40 ± 0.13 ng/ml) and we found the highest levels on day 13 p.i. (18.23 ± 0.64 ng/ml). These values represented a 7.7-fold increase with respect to the baseline levels. The animals were positive for anti-ML-ESP IgG2a until day 3 p.i.

Igs with specificity for the NBL surface were detected in the LTEs during the migration of this parasite through the lung and with the following pattern: on day 6 p.i., 13% of the animals presented specific IgA and IgE in undiluted LTEs; on day 13 p.i., 100% of the animals had a positive reaction for IgE and IgG1 (LTEs dilution $\leq$ 1:16), while only 50% of the animals presented IgA in undiluted LTEs and IgG2a (LTEs dilution $\leq$ 1:8).

Presence of Ig LSCs

The presence of total IgA, IgE, IgG1 and IgG2a LSCs in the lung tissue was observed throughout the study period, and we observed a significant increase in IgE and IgG1 LSCs ($P < 0.02$) on day 13 p.i. On day 3 p.i., we observed a significant decrease in the number of total IgA LSCs ($P < 0.03$).

Anti-ML-ESP IgA, IgE and IgG2a LSCs were found throughout the study period. We observed the presence of anti-ML-ESP IgG1 LSCs by day 13 p.i., together with an increase in the numbers of total IgG1 LSCs. We observed a significant decrease in the number of anti-ML-ESP IgA LSCs on day 13 p.i. ($P < 0.002$ and $P < 0.0008$ in comparison with days 3 and 6 p.i., respectively) (Table 2).

ADCC assay

The lung cells obtained from infected and control animals were able to adhere to and kill the NBL in the presence of the reference cytotoxic serum. The mortality percentages were 74.1 ± 10.4 and 53.8 ± 3.4 for the lung cells from infected and control rats, respectively. The reference cytotoxic serum was employed in all of the experiments as the positive control for cell activity.

The anti-NBL surface Abs present in the LTEs obtained on day 6 pi displayed basal mortality percentages 13.0 ± 1.0 and 8.0 ± 0.0 for the lung cells from infected and control rats, respectively. Only the LTEs obtained on day 13 p.i. that contained anti-NBL surface Abs were able to induce parasite death. The mortality percentages were 51.0 ± 1.4 and 42.5 ± 3.4 for the cells obtained from infected and control rats, respectively. These figures were not statistically different.

Discussion

Little is known about the immunopathological changes that occur in the lung during the first days of *T. spiralis* infection and the role of such changes in host protection (Dupouy-Camet and Bruschi 2007; Venturiello et al. 2007; Gottstein et al. 2009). The results obtained in this study indicated that during the first hours of infection with this nematode (intestinal phase), an intense cellular infiltrate comprised mainly eosinophils and mast cells in the lung parenchyma and hyperplasia in the BALT and goblet cells develop, together with the local secretion of IgE in the lung.

Taking into account the inflammatory process induced by the invasion of the pathogen in the intestinal mucosa (Knight et al. 2008; Suzuki et al. 2008), the simultaneous development of the lung inflammatory response found in our model might be attributed to the migration of the cells through the common mucosal immune system, which is dependent on chemotactic factors and/or inflammatory molecules. Cytokines IL-4 and IL-5 and chemokines CCL11 and CCL28, which are found in the mucosal bronchial milieu, might act as chemotactic factors. TNFα and INFγ are upregulators of CCL11 (Bartels et al. 1996; Lilly et al. 1997). These chemokines might recruit eosinophils and T cells by binding and signaling pathways involving CCR3 and CCR10 (Humbles et al. 2002; John et al. 2005; Dixon et al. 2006).

The levels of IL-10 in the experimental animals were significantly higher (Fig. 4) than those of control animals when the NBL arrive in the lung on approximately day 5 p.i. (Denham and Martínez 1970; Harley and Gallicchio 1971). Such upregulation of IL-10 was accompanied by falls in the levels of TNFα, CCL11 and CCL28, suggesting the establishment of an immunomodulatory mechanism. This immunomodulatory effect was also reflected in the temporary decrease in anti-ML-ESP IgE and anti-ML-ESP IgG1 levels on day 6 p.i., followed by a rise in the levels of total IgE and total IgG1 (Fig. 5) concomitantly with the development of a Th2 adaptive immune response. The fall in the levels of anti-ML-ESP IgE and anti-ML-ESP IgG1 might occur due to the secretion of

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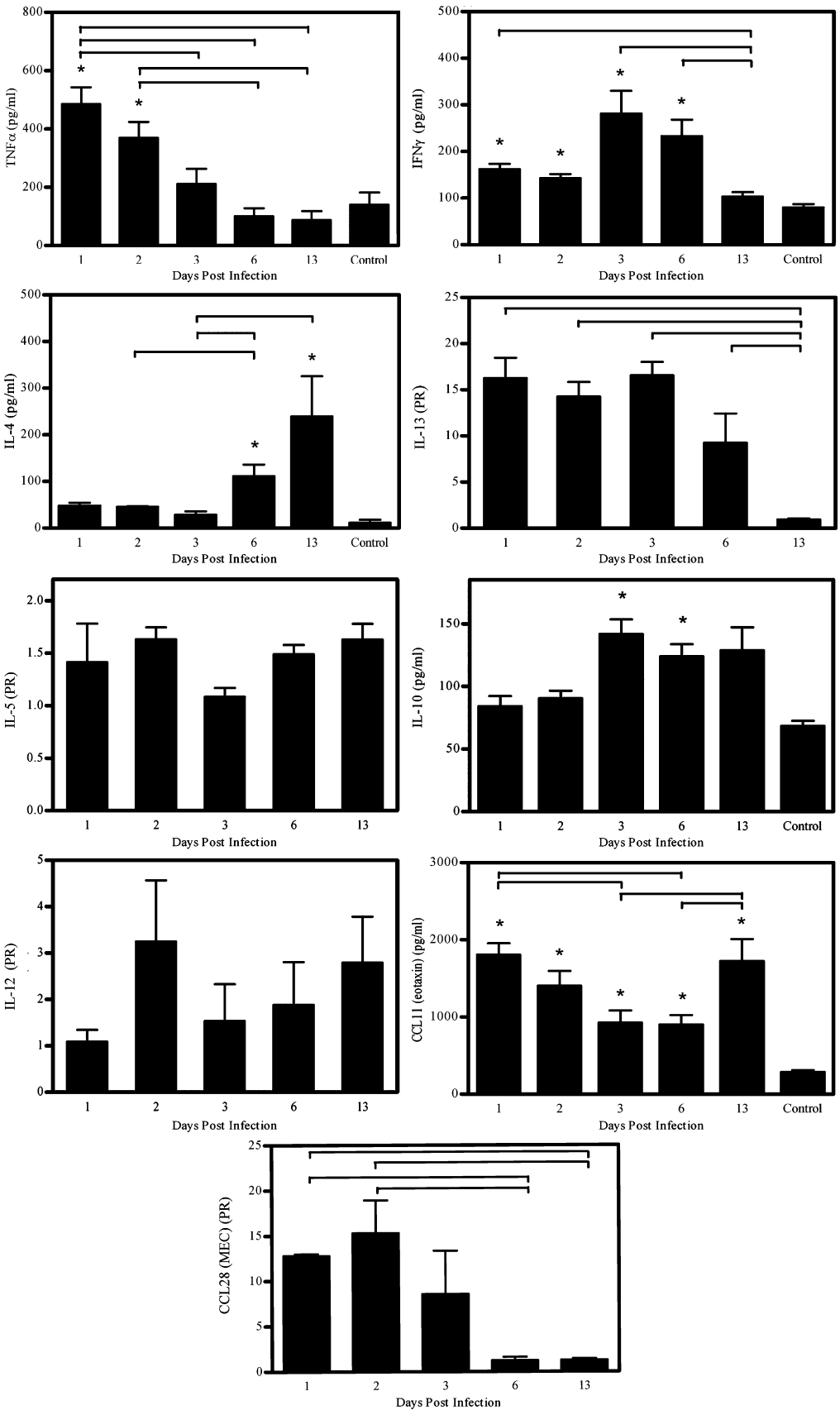

Fig. 4. Cytokine and chemokine levels in LTEs ($n = 5$/days p.i.) during the early phase of *T. spiralis* infection. The concentrations (ng/ml) of INFγ, TNFα, IL-10, IL-4 and CCL11 were determined by commercial ELISA kits. The IL-5, IL-12, IL-13 and CCL28 were determined by immunoelectrotransfer blot assay and expressed as positivity ratios (PR) = [ODcytokine band/ODβ-actin band] infected/average [ODcytokine band/ODβ-actin band] control animals. Reactions were considered positive when the PR > 1. The values shown represent the mean of concentration and PR ± the SEM and are representative of 2 independent experiments. Asterisks indicate significant differences between infected and control animals (TNF and IL-13: $P \ll 0.05$, IL-10: $P = 0.004$, two-way ANOVA test; IL-4: $P = 0.003$, CCL11: $P = 0.0002$, IFNγ: $P = 0.0001$, non-parametric Kruskal–Wallis ANOVA, CCL28: $P = 0.001$, one-way ANOVA). Bars indicate significant differences among the indicated days.

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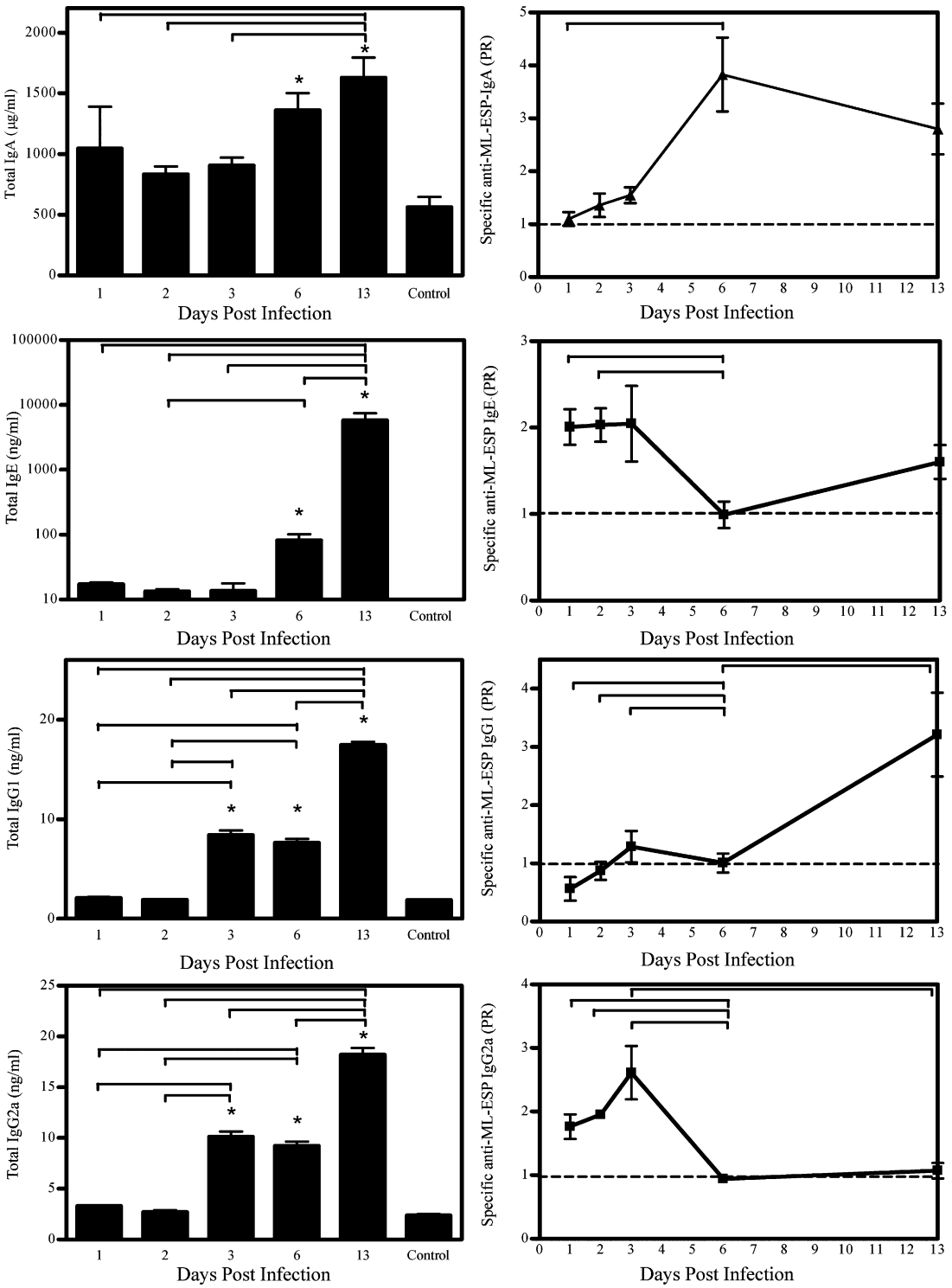

Fig. 5. Levels of total Ig isotypes and detection of specific Igs in LTEs ($n = 5$/days p.i.) during the early phase of *T. spiralis* infection. The concentrations of total Igs were determined by commercial ELISA kits. The levels of specific Igs were determined by ELISA and were expressed as PR = OD of infected animals' LTEs/OD of control animals' LTEs. A PR > 1 was considered positive. The values shown represent the mean levels and the PR ± the SEM. The asterisks indicate the significant differences between the infected and control animals (specific IgA: $P = 0.002$, specific IgE: $P = 0.05$, total IgG1 and total IgG2a: $P \ll 0.05$, two-way ANOVA test; total IgA and total IgE: $P \ll 0.05$, specific IgG2a: $P = 0.014$, specific IgG1: $P = 0.001$, by the non-parametric Kruskal–Wallis ANOVA). The bars indicate significant differences among the indicated days. The results are representative of two experiments.

immunomodulatory factors by NBL as an evasion mechanism and may be part of the regulatory process of the host-parasite relationship. The immunosuppression phenomena related to the secretion of such factors have already been demonstrated (Faubert 1976, 1982) or suggested by other authors (Dzik et al. 2002). Another mechanism involved in the suppression of such responses might also occur due to a cross-reactivity phenomenon between the NBL Ags and the ML-ESP (Venturiello et al. 1996, 2000; Malmassari et al. 2003), and the Abs may be consumed by opsonization by the NBL. It is noteworthy that the number of NBL in the lung cell suspensions was higher on day 6 p.i. than that found on day 13 p.i., indicating a greater inflow of parasites in the lung and therefore, greater

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Table 2
Number of total and specific immunoglobulins lung secreting cells (Ig LSCs) in lung tissue during the intestinal phase of *Trichinella spiralis* infection.

Days p.i.	IgA		IgE		IgG1		IgG2a	
	Total	Anti ESP-ML	Total	Anti ESP-ML	Total	Anti ESP-ML	Total	Anti ESP-ML
3	2211 ± 313^{b}	29 ± 10^{c}	2 ± 1	2 ± 1	170 ± 38	0 ± 0	165 ± 56	1 ± 1
6	3511 ± 386	37 ± 14^{d}	5 ± 1	5 ± 1	93 ± 29	0 ± 0	104 ± 44	7 ± 4
13	3455 ± 97	5 ± 2^{e}	10 ± 3^{f}	9 ± 3	553 ± 23^{h}	40 ± 9	347 ± 32	8 ± 2
Control	3742 ± 339^{a}	0 ± 0	1 ± 0^{e}	0 ± 0	227 ± 38^{g}	0 ± 0	236 ± 82	0 ± 0

Data are expressed as mean ± S.E.M. of number Ig LSCs/10^6 lung leukocytes obtained from 3 independent experiments performed with groups of 5 animals/day p.i. Significant differences between infected and control rats: $P < 0.002$: c *vs.* e; $P < 0.008$: d *vs.* e; $P < 0.02$: f *vs.* e, h *vs.* g; $P < 0.03$: b *vs.* a. Data were analyzed by a two-way ANOVA test.
ESP-ML: muscle larvae excretory–secretory products.

consumption of anti-ML-ESP Abs. Surprisingly, this phenomenon is not evidenced with specific IgA, suggesting the absence of Ig reactivity toward the epitopes shared between the stages of development.

Although high levels of total IgA in the LTE were found in rats on days 6 and 13 p.i., this increase was not observed in the number of IgA LSC and the number of IgA^+ cells in the BALT, suggesting that the synthesis rate of Igs was superior to the secretion. These results are in line with those of Wang et al. (1999), who demonstrated that the number of activated B cells is not necessarily correlated with the amount of Ab molecules produced.

The significant decrease in the number of IgA^+ cells present in the BALT on day 1 p.i. may be correlated with a class switching phenomenon from IgA to other Ig isotypes, most likely IgE because on day 2 p.i. we observed an increase in the number of IgE^+ cells in 100% of the infected animals.

Although the basal number of IgA^+ cells was greater than the number of IgE^+ cells in the BALT, the latter underwent an abrupt increase during the infection. The increase in the number of IgE^+ cells in the BALT, together with the detection of anti-ML-ESP, the anti-NBL surface IgE found in the LTE and the presence of IgE LSCs in the lung tissue, clearly demonstrate the local production of this Ig in response to the infection.

Taking into account that the gut is the first location of *Trichinella* antigenic stimuli, the very early detection of specific Igs in the lung (anti-ESP-ML on day 1 p.i. and anti-NBL surface on day 6 p.i.) might be due to the migration of activated B cells from the gut into the lung. Gut T cells are activated as early as 12 h p.i. in rats infected with *T. spiralis*, and they interact with B cells to produce Abs in the small intestine shortly after the infection (Wang et al. 1999; Wagner et al. 2004).

The results of the immunological parameters associated with the detection of specific Abs in the LTEs were corroborated by experiments conducted with SPF (specific pathogen free) Wistar rats to ensure the specificity the detected anti-*Trichinella* Abs. The results of the different Ab isotypes studied in SPF Wistar rats showed the same detection profile: (a) the presence of all the isotypes studied; (b) the detection of IgA, IgE and IgG2a anti-ESP-MLs from day 1 p.i. (PR = 1.1 ± 0.1, 2.0 ± 0.2 and 1.8 ± 0.2, respectively) and specific IgG1 on day 3 p.i. (PR = 1.3 ± 0.2); (c) the peak values of specific IgA were found on day 6 p.i. (PR = 3.8 ± 0.7), the peak specific IgE on days 1 and 2 p.i. (PR = 2.0 ± 0.2, 2.0 ± 0.1, respectively), the peak specific IgG1 on day 13 p.i (PR = 3.2 ± 0.7) and the peak specific IgG2a on days 1, 2 and 3 p.i. (PR = 1.8 ± 0.2, 2.0 ± 0.0 and 2.6 ± 0.4, respectively); and (d) the day p.i. on which NBL are detected in lung, there is a decrease in the levels of specific IgE and IgG1 (day 6 p.i. PR ≤ 1.0). With regard to the anti-surface NBL Abs, the specific IgA and IgE were detected on day 6 p.i., and the IgE, IgA, IgG1 and IgG2a were detected in the LTEs on day 13 p.i.

The role of the IgE might be, on the one hand, to maintain an anaphylactic reaction with the subsequent mast cell degranulation, leading to the secretion of histamine, IL-4 and chemotactic substances, which are responsible for the accumulation of inflammatory cells. On the other hand, this Ig plays a role in the adaptive immune response, acting as a link between the effector cells (leukocytes) and the parasite to mediate ADCC reactions (Bruschi et al. 1992; Venturiello et al. 1995). In addition, the latter effector mechanism might be enhanced by the lung architecture. This hypothesis is supported by the finding of the migratory-stage pathogen in both the lung parenchyma and alveoli and by the helminthocytotoxic activity of the lung cells, which were able to induce NBL death through the action of anti-NBL surface Abs.

The continuous presence of IgE at concentrations higher than 1 μg/ml stimulates the IgE-sensitized mast cells to activate and release soluble factors in an Ag-independent manner (Mitre and Nutman 2006). In fact, the degranulated mast cells were observed in the histological sections (Fig. 1D). Our results suggested a crucial role of the mast cells in the lung inflammatory reaction by contributing to the amplification of the protective response generated against *T. spiralis*.

Upon evaluating the biological activity of the lung cells, we observed that the cells obtained on day 6 p.i. were able to kill the NBL in the presence of specific Abs present in both the cytotoxic serum (mortality percentages 74.1 ± 10.4 and 53.8 ± 3.4 for the lung cells from infected and control rats, respectively) and the LTEs (the mortality percentages of LTE obtained on day 13 p.i. were 51.0 ± 1.4 and 42.5 ± 3.4 for cells from infected and control rats, respectively; the LTEs obtained on day 6 p.i. showed basal values of mortality percentages 13.0 ± 1.0 and 8.0 ± 0.0 for the lung cells from infected and control rats, respectively). Although no significant differences were observed in the mortality percentages of the NBL in ADCC reactions using cells of infected rats or controls, we observed a higher trend of helminthocytotoxic activity in reactions using cells from infected rats.

The Abs present in the LTEs obtained on day 13 p.i. had helminthocytotoxic activity, probably due to the higher effector Ab titers found in these samples. These were mainly the anti-NBL surface IgE and IgG1 (LTEs dilution on day 13 p.i. was $\leq$ 1:16, while the LTE on day 6 p.i. was undiluted), reinforcing the effector role of these Igs in *T. spiralis* infection (Wang and Bell 1992; Venturiello et al. 1995).

Our observations indicated that the intestinal infection with *T. spiralis* induces an early signal in the lungs in three phases. The initial phase (days 1 through 3 p.i.) is related to the mucosal inflammatory processes involved in the innate defense mechanisms and the mucosal compartmentalization phenomenon, with the presence of IgE, IgA, chemokines and cell infiltrates. This phase is followed by a second phase (on days 3–6 p.i.), which shows a mixed Th1/Th2-type response with the presence of IL-10. Finally, the Th2-skewed response (on days 6–13 p.i.) is probably driven by the presence of migratory larvae and is characterized by the presence of maximum eosinophilic infiltrates; then, there is a predominance of CCL11, IL-4 and IL-5, with high levels of total IgE > IgG1 > IgG2a > IgA, together with the detection on day 13 p.i. of anti-NBL surface Abs of all the isotypes studied.

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In summary, the following conditions were fulfilled: (1) the compartmentalization phenomenon, particularly between intestinal and bronchial mucosae resulted in sensitized lung tissue that is generated prior to the arrival of the parasite to the organ; (2) both the lung cells and the anti-NBL surface Abs produced locally had helminthocytotoxic activity; and (3) during the migration to the striated muscle, the NBL found in the lung parenchyma and alveoli are opsonized along with bloodstream and tissue Abs, thus activating the effector cells to mediate parasite death during its stay in the lung. Upon arrival at the lungs, the parasite encounters a reactive organ that acts as a retention and parasite destruction site. Only those larvae capable of avoiding this response will achieve muscle invasion (Gansmüller et al. 1987; Bruschi 2002). Thus, during *T. spiralis* infection, not only an effector mechanism directed toward adult worms in the intestine is developed, but also our results suggest an effector mechanism directed against the migrant stage in the lung.

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