

Immunohistochemical detection of GM-CSF, IL-4 and IL-5 in a murine model of allergic bronchopulmonary aspergillosis

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Summary

Background Granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-4 (IL-4) and IL-5 are important in tissue eosinophil accumulation and high IgE production in allergic inflammatory reaction.

Objective We examine lung GM-CSF, IL-4 and IL-5 expression in a murine model of allergic bronchopulmonary aspergillosis (ABPA) characterized by eosinophil and lymphocyte lung infiltration and elevated serum IgE level.

Methods C57BL/6 mice were intranasally treated three times a week for 1, 2 or 3 week(s) with *Aspergillus fumigatus* (Af) antigen or saline and were sacrificed on days 7, 14 and 21. Immunohistochemical analyses for GM-CSF, IL-4 and IL-5 were performed on lung sections.

Results Af treatment induced a remarkable pulmonary eosinophil influx. Increased numbers of lung T lymphocytes and GM-CSF positive cells were observed on days 14 and 21. IL-4 and IL-5 positive cells were increased significantly only on day 14. Immunostained serial sections showed that most ($\geq 98\%$) cytokine positive cells were CD3 positive. Few eosinophils ($< 2\%$ of cytokine positive cells) were immunoreactive for GM-CSF and IL-5. Significant correlations were found between the number of GM-CSF and IL-5 positive cells, and the number of eosinophils in Af-treated lung ($r = 0.62$, $P < 0.05$ and $r = 0.52$, $P < 0.05$, respectively), and between the number of IL-4 positive cells and the serum total IgE level ($r = 0.64$, $P < 0.01$).

Conclusions Our data suggest a role for T lymphocyte GM-CSF, IL-4 and IL-5 in Af-induced mouse pulmonary eosinophilia and increased serum IgE production and further support the importance of T helper (TH₂) cells in the pathogenesis of ABPA.

Keywords: ABPA, eosinophils, GM-CSF, IL-4, IL-5, T lymphocytes, IgE, lung, immunohistochemistry, mice

Clinical and Experimental Allergy, Vol. 26, pp. 461–468. Submitted 4 November 1994; revised 8 August 1995; accepted 19 October 1995.

Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity disease of the lung caused mostly by the inhalation of *Aspergillus* species. Patients with ABPA show blood and pulmonary eosinophilia and elevated serum IgE level [1–4]. Recent studies indicated that activated T lymphocytes, especially T-helper 2 (TH₂)

cells, are involved in airway allergic inflammation [5–6]. TH₂ cells secrete high levels of IL-4, IL-5, IL-6, IL-10, IL-13, and also IL-3, GM-CSF and TNF α [7]. IL-4 acts as switching signal for IgE synthesis [8]. The cytokines IL-3, IL-5 and GM-CSF may play a pivotal role in recruiting and activating immune effector cells, especially eosinophils [9]. IL-5 appears to be the most specific cytokine for eosinophils. It promotes the terminal differentiation of committed eosinophil precursors, enhances eosinophil adhesion to endothelial cells, activates mature

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eosinophils and prolongs their survival in target tissues. Several studies demonstrated that pulmonary eosinophilia in animal models can be blocked by antibodies against IL-5 [10,11].

To explore the pathogenesis of human ABPA, a murine model of ABPA characterized by pulmonary eosinophilia and elevated serum IgE level has been reported by Kurup *et al.*, and also by our team [12–16]. Kurup *et al.* [12–14] found increased IL-4, IL-5 and IL-10 production in the supernatants of Af antigen stimulated lung lymphocytes obtained from mice exposed to Af antigen. Anti-IL-5 and anti-IL-4 abrogated blood and lung eosinophilia, and IgE hypersecretion, respectively. In the present study, we examined the expressions of IL-4, IL-5 and GM-CSF in the lung tissue by immunohistochemistry and identified the cells expressing these cytokines.

Materials and methods

Animals

Twenty-seven pathogen-free female inbred C57BL/6 mice of 6–8 weeks old were obtained from Charles River Inc. (St-Constant, Québec, Canada) and randomized before the sensitization.

Induction of ABPA

Induction of ABPA was performed as previously described [15,16]. Briefly, the mice were lightly anesthetized with diethyl ether before 50 mL of *Aspergillus fumigatus* antigen (100 mg in dry weight, Af group) or 50 mL saline (Sa group) was administered to the tip of the nose and inhaled involuntarily. The inhalations were given three times a week during the first three successive days of each week for 1, 2 or 3 week(s). On days 7, 14 and 21, mice were sacrificed by exsanguination. At each time point, five mice were included in Af group and four in Sa group.

Lung tissue processing

The whole lungs were removed from the thoracic cavity. They were immediately dissected into small pieces of approximately $2 \times 2 \times 1$ mm. For each mouse, two to four pieces of tissues were randomly selected from the left and right lungs and processed as reported by Britten *et al.* [17]. Briefly, lung tissues were placed in ice-cooled acetone containing protease inhibitors, phenylmethylsulfonyl fluoride (2 mM) and iodoacetamide (20 mM), and stored overnight at -20°C . The following day, lung tissues were transferred into acetone for 15 min and

methyl benzoate for another 15 min at room temperature, followed by an immersion in glycol methacrylate (GMA) monomer (Polyscience Inc., Warrington, PA, USA) at 4°C for 7 h. Finally, the tissues were embedded in the resin prepared by mixing 10 mL GMA monomer with 25 mL benzoyl peroxide and 45 mg N, N-dimethylaniline, and polymerized overnight at 4°C . The blocks were stored in airtight containers at -20°C for later histochemical and immunohistochemical analyses.

Histochemistry

For routine histological evaluation, the sections were stained with haematoxylin and eosin. Eosinophils in the tissue were specifically identified by 1% chromotrope 2R staining [18]. Mast cells and or basophils were identified by toluidine blue (pH 1.0) staining.

Immunohistochemistry

Sequential 2 mm sections were cut with an ultramicrotome (Reichert Ultracut S, Vienna, Austria), placed on glass slides coated with poly-L-lysine and dried at room temperature. To block endogenous peroxidase, the slides were placed in 0.1% sodium azide and 0.3% hydrogen peroxide solution for 30 min and rinsed in 0.05 M Tris-buffered saline (TBS, pH 7.6). Successive incubations in avidin for 20 min and biotin for 20 min (Blocking Kit, Vector Laboratories, Burlingame, CA, USA) were then performed to eliminate the endogenous biotin activity in the lung. To prevent the non-specific protein binding, 10% normal rabbit serum was applied to the sections for 30 min before adding the primary antibodies. Monoclonal rat anti-mouse CD3, CD4, CD8 (PharMingen, San Diego, CA, USA), IL-4, IL-5 and GM-CSF (Genzyme, Cambridge, MA, USA) antibodies were used at dilution of 1:50. Sections were incubated with these antibodies overnight at room temperature. Biotinylated rabbit secondary antibody diluted in TBS containing 2% normal mouse serum was then applied for 2 h followed by the streptavidin-biotin-horseradish-peroxidase complex (Sera Lab, Sussex, UK) for another 2 h. After rinsing the sections in TBS, 0.01% diaminobenzidine (DAB) in 0.02% hydrogen peroxide was used as substrate to develop a peroxide-dependent brown color reaction. All the sections were counterstained with Harris haematoxylin and coverslipped with Permount. Appropriate unrelated antibodies of the same isotypes and concentrations as the primary monoclonal antibodies were applied to the negative control sections.

Measurement of serum total IgE

Serum total IgE was determined by enzyme linked

immunosorbent assay (ELISA) as described previously [15]. All samples were analysed in duplicate. The results were expressed in ng/mL.

Analyses of histochemical and immunohistochemical sections

Lung sections were coded and counted blindly using a Leitz Wetzlar microscope at $\times 200$ magnification field (0.62 mm^2). Because the distribution of immunostained cells and eosinophils was not uniform, the entire section (≥ 5 fields per section) was counted. Counts were expressed as the number of cells per $\times 200$ microscopic field. For each mouse, two to four sections were examined and a mean of all the examined fields was given for each parameter. For mice sacrificed at the same time point in Af or Sa group, we calculated a mean $\pm$ SEM for each parameter. Each section was counted at least twice.

The coefficient of variation (CV) for repeated measurement was $\leq 7\%$.

Statistical analyses

Student's *t*-test was applied to the comparisons between Af and Sa groups. For the comparisons within the same group at different times, Fisher's test was used, followed by Tukey's comparison. Correlations between various groups of data were carried out using Spearman's rank correlation coefficient. A *P*-value of ≤ 0.05 was regarded as significant.

Results

Histological findings

Mice of Sa group showed normal lung structure without

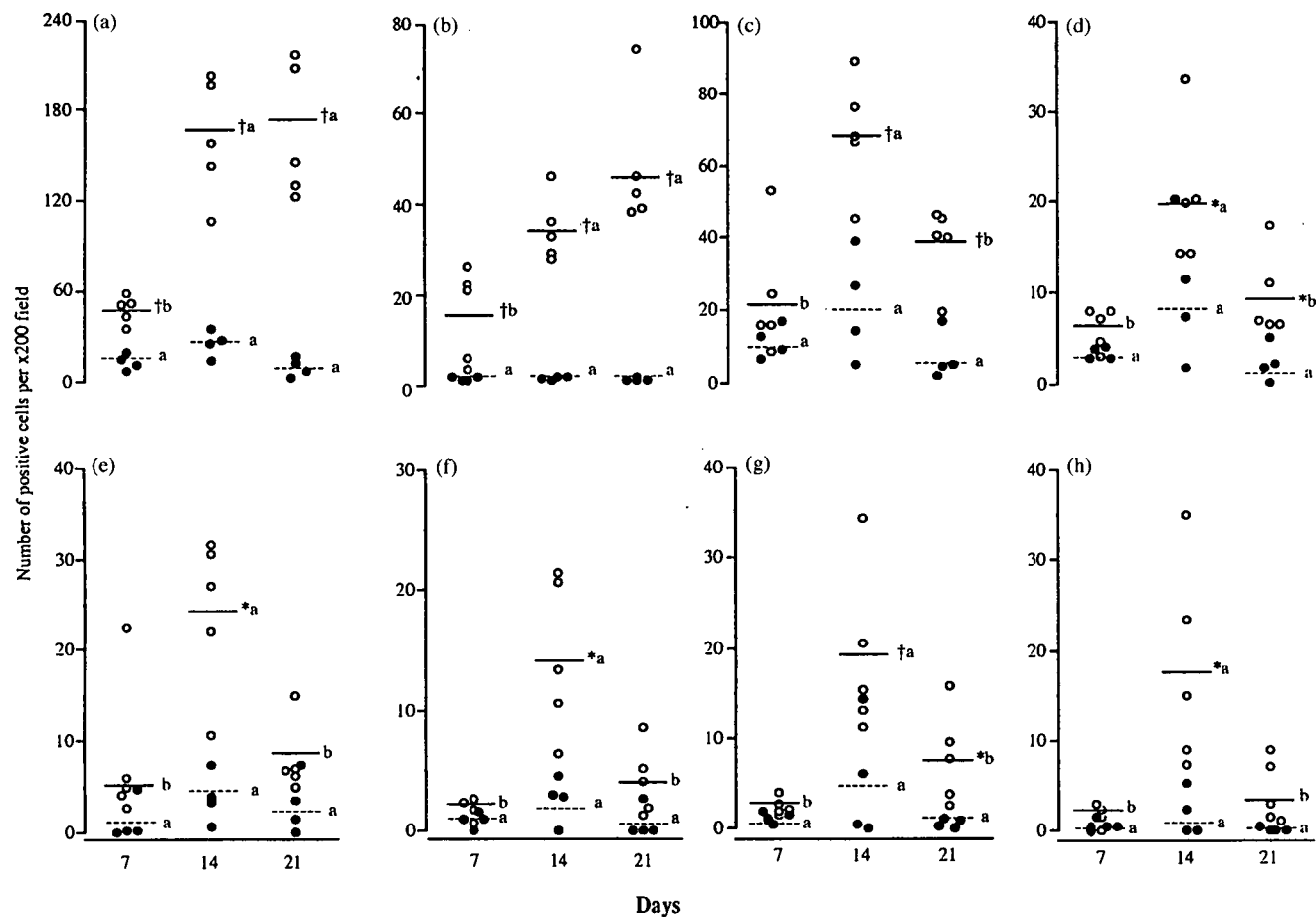


Fig. 1. (a) Total inflammatory cells, (b) eosinophil and (c) CD3, (d) CD4, (e) CD8, (f) IL-5, (g) GM-CSF and (h) IL-4 positive cell counts on days 7, 14 and 21. Each dot (○ Af group, ● Sa group) represents the mean result from an individual mouse lung tissue. The continuous and broken cross bars represent the mean of Af and Sa groups, respectively. * $P < 0.05$ and † $P < 0.01$, Af group vs Sa group. Comparisons were also made within either Af or Sa group between days 7, 14 and 21 and values identified by a different letter are significantly different ($P < 0.05$).

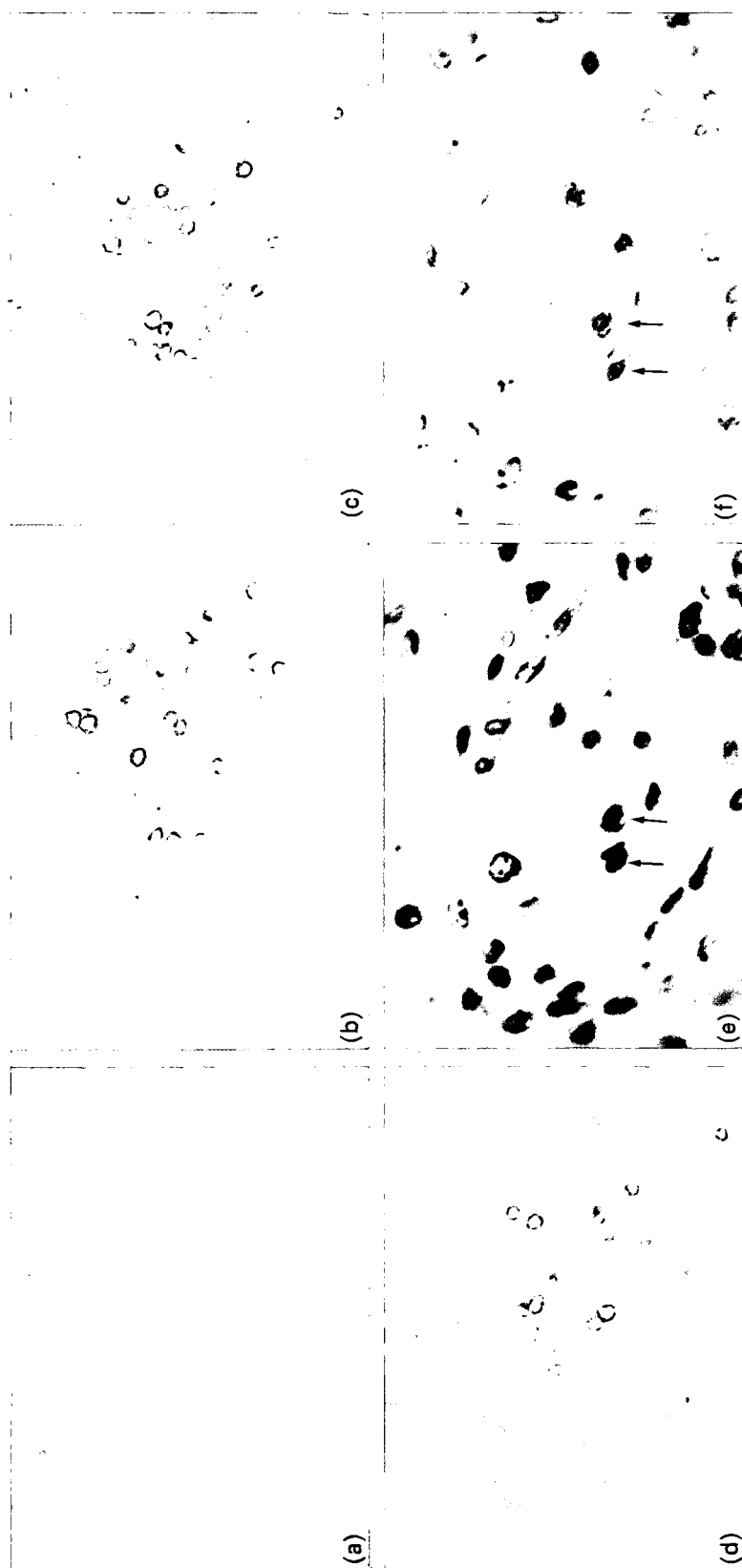


Fig. 2. Lung tissue preparations of Af-treated mice on day 14. (a–d) are serial sections. a there is no staining in a negative control section incubated with non-specific rat IgG1. (b), (c) and (d) respectively show GM-CSF, CD3 and IL-4 stained cells around a small blood vessel and a bronchiole. (e) and (f) are serial sections. (e) shows some IL-5 stained cells (arrows) and (f) (chromotrope 2R staining) demonstrates that most IL-5 positive cells seen in (e) are eosinophils (arrows). Original magnifications: (a–d), $\times 400$; (e–f) $\times 1000$.

any evidence of inflammatory lesions. In contrast, all Af-challenged mice demonstrated an interstitial pneumonitis with various degrees of eosinophil infiltration (Fig. 1). On day 7, increased numbers of eosinophils, monocytes and alveolar macrophages were dispersed throughout the lung interstitium and air spaces. These inflammatory cells were predominantly localized around small blood vessels and bronchioles. On day 14, peribronchiolar and perivascular cellular infiltrate was more obvious. Numerous eosinophils, macrophages and monocytes infiltrated the peribronchiolar and perivascular spaces, alveolar walls and air spaces. On day 21, the lung lesions were more severe. This remarkable inflammatory cell infiltration resulted in local lung structure distortion. In some mice, epithelioid granuloma with giant cells could be observed at this time.

Serum total IgE measurement

The serum total IgE level was low on day 7 and there was no significant difference between Af and Sa groups. Compared with saline-treated mice, significant increases of serum total IgE level were found in Af-challenged mice on day 14, 9941 ± 530 vs 50 ± 5.0 ng/mL ($P < 0.001$), and on day 21, 5633 ± 581 vs 40 ± 4.7 ng/mL ($P < 0.001$).

Immunohistochemical analyses

No significant staining was observed in any of the negative control sections (Fig. 2a). The immunohistochemical counts of lung tissue CD3, CD4, CD8, GM-CSF, IL-4 and IL-5 positive cells are shown in Fig. 1. Compared with control mice, the number of CD3, CD4, CD8, GM-CSF, IL-4 and IL-5 positive cells significantly increased in Af-treated mice on day 14, but not on day 7. On day 21, only CD3, CD4 and GM-CSF positive cell numbers were significantly increased.

Most immunostained cells were present around bronchioles and small blood vessels and some were also observed in the alveolar septa. Immunostaining for cytokines was localized in the cytoplasm. Staining of serial 2 mm sections showed that almost all GM-CSF, IL-5 and IL-4 positive cells were CD3 (Fig. 2b, c and d) and CD4 (Fig. not shown) positive. Among cytokine positive cells, T cells represent 98.1% (± 0.8) for GM-CSF, 100% for IL-4 and 99.5% (± 0.4) for IL-5. To

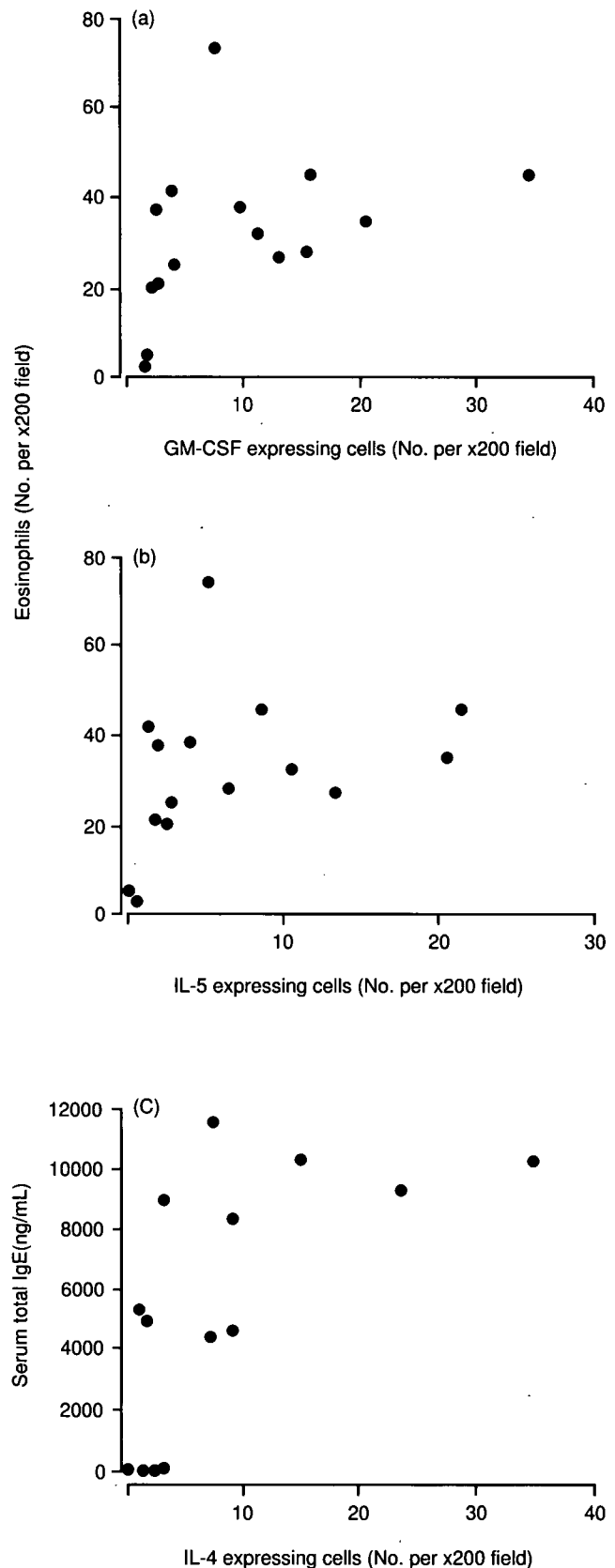


Fig. 3. Correlations between GM-CSF (a) or IL-5 (b) expressing cells and eosinophils and between tissue IL-4 (c) expressing cells and serum total IgE level within Af group at all the examined time points. (a) $r = 0.62$, $P = 0.013$. (b) $r = 0.52$, $P = 0.045$. (c) $r = 0.64$, $P = 0.009$.

identify the cell types of remaining cytokine positive cells, we performed chromotrope 2R staining for eosinophils and toluidine blue staining for mast cells and/or basophils in the sequential sections or in the cytokine stained sections. We found very few mast cells or basophils in the lung tissue, they were usually seen around main branches of bronchi and large and middle-sized blood vessels. None of these mast cells or basophils were positive for GM-CSF, IL-4 and IL-5. However, we did find that some eosinophils expressed GM-CSF ($1.9 \pm 0.7\%$ of GM-CSF+ cells) and IL-5 ($0.5 \pm 0.3\%$ of IL-5+ cells) (Fig. 2e and f). We did not find any bronchial epithelial cells expressing GM-CSF. We also determined the percentages of cytokine-expressing T cells. We found that 19.4% (± 5.1) of T cells were positive for GM-CSF, 14.3% (± 6.1) for IL-4 and 12.4% (± 4.1) for IL-5.

Correlations between cytokine-expressing cells, eosinophils and serum IgE level

In Af-challenged mouse lungs, the number of cells expressing GM-CSF and IL-5 correlated significantly with the number of eosinophils ($r = 0.62$, $P = 0.013$ and $r = 0.52$, $P = 0.045$, respectively) (Fig. 3a and b). Significant correlations were also found between serum total IgE level and the number of IL-4 ($r = 0.64$, $P = 0.009$) (Fig. 3c) as well as IL-5 ($r = 0.71$, $P < 0.001$) expressing cells.

Discussion

In human, ABPA induces an important recruitment of eosinophils into the lung and a dramatic increase in immunoglobulin production, especially IgE. These features are believed to be involved in the pathogenesis of this disease. We observed similar alterations in our murine model of ABPA. Since TH₂ lymphocytes can induce both IgE synthesis and eosinophil recruitment and activation, it is tempting to postulate that they participate in the inflammatory events of our murine ABPA model. In this study, we demonstrated that pulmonary eosinophilia and high serum IgE level are related to TH₂ lymphocyte infiltration in the mouse lung tissue.

The number of cells immunoreactive for GM-CSF, IL-4 and IL-5 increased in Af-treated mice, although it varied at the different time points examined. In immunohistochemistry, the best way to identify the cell source of cytokine expression at the light microscopic level is to do a double immunostaining for cytokine and a specific cell marking on the same section. In our hands, this technique failed to provide good stainings since the two

different color reactions were difficult to distinguish, especially on lymphocytes with small cytoplasm. Therefore, we embedded the lung tissue in GMA resin and cut it into 2 mm serial sections for immunostaining. This technique allowed us to analyse the same cell in three or four different sequential sections and provided valuable results. We showed that most IL-4, IL-5 and GM-CSF positive cells were T cells. Previous studies demonstrated that besides T cells, other cells can also synthesize these cytokines. IL-4 can be produced by mast cells, IL-5 by mast cells and eosinophils, and GM-CSF by macrophages, eosinophils, mast cells, alveolar and bronchiolar epithelial cells, and fibroblasts [18–21]. However, our results showed that among these non-T cells, only few eosinophils were positive for GM-CSF and IL-5. This finding may suggest that the cell source of cytokine GM-CSF, IL-4 and IL-5 detected by immunohistochemical staining is limited to T cells and eosinophils in this ABPA mouse model.

We also found some cytokine expressing cells in the lung of control mice. On day 14, there was a slight but not statistically significant increase in the number of T lymphocytes and cytokine positive cells. In our protocol, control mice involuntarily inhaled the saline. We believe that not only the saline but also some secretions of the upper respiratory tract enter the lung at the same time. These secretions may include mucus, cellular debris and possibly some normal residential bacteria and viruses which may initiate a slight lung immunological response and result in an increased number of T lymphocytes, and cytokine expressing cells in the lung tissue of control mice.

Wynn *et al.* [22] described the kinetics of various cytokine murine ribonucleic acid (mRNA) expressions in the mouse lung during granuloma formation induced by *Schistosoma mansoni* eggs. They showed that eosinophil accumulation and increased serum total IgE level were most obvious on day 15, and that IL-5, IL-4 and GM-CSF mRNA expressions peaked on day 6, and declined on day 15. They did not detect the protein expression of these cytokines in the tissue. Our present study demonstrated an important increase in the numbers of GM-CSF, IL-4 and IL-5 expressing cells in the lung of Af-treated mice on day 14. By day 21, only GM-CSF expressing cells were significantly increased. The fact that on day 7 lung tissue of Af group showed a significant increase of eosinophil influx without significantly increased GM-CSF and IL-5 expression may be due to the time point we examined after antigen challenge. We postulate that before day 7, the fourth day after the last antigen challenge of the first week, there was a peak of cytokine production which was essential for the lung eosinophil recruitment and by day 7, the cytokines

were already released from most cytokine-producing cells which were then negative by immunohistochemical detection. Concerning the intensity of eosinophil influx in the lung tissue, no significant difference was found between days 14 and 21, but less T cells, GM-CSF and IL-5 positive cells were observed on day 21. We speculate that tissue eosinophilia may be mainly driven by TH₂ cells on day 14. On day 21, tissue eosinophil influx may be orchestrated by cytokines not only from TH₂ cells, but also from other types of cells such as eosinophils and mast cells whose cytokine production may be too low to be detected by immunohistochemistry.

In this study, we were able to establish a strong correlation between *in situ* GM-CSF and IL-5 expression and pulmonary eosinophilia in Af-treated mice. These findings suggest that in murine ABPA model, local lymphocyte GM-CSF and IL-5 expressions are involved in tissue eosinophil accumulation and further demonstrate the important roles of TH₂ lymphocytes and their cytokines and eosinophilic inflammation.

Immunoglobulin isotype switching to IgE synthesis is mainly mediated by IL-4 and costimulatory signals provided by CD4⁺ T cells. Several cytokines modulate IL-4-dependent IgE production. Interferon (IFN) α , IFN γ , TGF β , IL-8, IL-10 and IL-12 have inhibitory effects, whereas IL-2, IL-5, TNF α and IL-6 enhance IL-4-induced IgE synthesis [23–25]. We found that tissue IL-4 and IL-5 expressing cells increased in number and peaked on day 14, which was similar to the profile of serum total IgE production. Both tissue IL-4 and IL-5 expressing cells correlated with serum total IgE level. This suggests that IL-4, along with IL-5, could be a major modulator of IgE production in this mouse ABPA model.

In conclusion, our study suggests that T lymphocytes, especially TH₂ subsets, are involved in the pathogenesis of murine ABPA model. In the present study, we detected GM-CSF, IL-4 and IL-5 by immunohistochemistry. To further elucidate the pathogenesis of ABPA, future work will be aimed at examining the importance of other cytokines which could also be involved in eosinophil recruitment and IgE production, such as IL-3, IL-8, IL-10, IL-13 and RANTES [26–28], and looking for more types of cytokine-producing cells at the tissue level by using combination of *in situ* hybridization technique and immunohistochemistry.

Acknowledgments

The authors are grateful to Serge Simard for his statistical analysis assistance. This study has been supported by a grant from the MRC of Canada (10032).

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