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Bronchopulmonary hyperreactivity and lung eosinophil sequestration but not their migration to the alveolar compartment are independent of interleukin-5 in allergic mice



Abstract

IL-5 is present in the lung and in the circulation following allergenic challenges in humans and in animals, but its role in bronchopulmonary hyperreactivity (BHR) and lung and bronchoalveolar lavage fluid (BALF) eosinophilia remains unclear. Because compartmentalization of IL-5 is recognized, the anti-IL-5 monoclonal

antibody TRFK-5 or its isotype control GL113 were delivered selectively intranasally (i.n.) and/or intravenously (i.v.) before the prior i.n. challenge with 10 µg OVA in BALB/c and BP2 "Biozzi" mice immunized according to optimized protocols with read-outs taken 24 h later. IL-5 in the BALF was suppressed by i.n. TRFK-5, whereas its production persisted in the serum. Conversely, i.v. TRFK-5 suppressed IL-5 in the serum but not in the BALF. IL-5 was suppressed in conditioned medium from lung explants from mice treated with i.n. TRFK-5, which did not affect the other Th2 cytokines, IL-4 and IL-13. IL-5 is thus present in the alveolar, pulmonary and circulatory compartments following an i.n. allergenic challenge. When specific anti-IL-5 antibodies were delivered by the same i.n. route, BALF eosinophilia was markedly reduced, whereas BHR and lung eosinophil sequestration persisted totally or mostly, in both strains. The passage of eosinophils from lungs to alveoli depends on IL-5 released into the BALF, but not into circulation, whereas their lung sequestration and BHR are mostly IL-5-independent. IL-5 alone does not account for the complexities of BHR or of eosinophil tissue trapping, and lung-targeted immunobiologicals should be delivered into the appropriate compartment in order to assess the role of specific mediators in experimental airways/lung allergy.

Full text

ABBREVIATIONS

AUC: area under the curve; BALF: bronchoalveolar lavage fluid; BHR: bronchopulmonary hyperreactivity; ELISA, enzyme-linked immunosorbent assay; EPO: eosinophil peroxidase; i.n., intranasally; i.v., intravenously; LPS: lipopolysaccharide; Mch: methacholine; mAb: monoclonal antibody; OD: optical density; OVA: ovalbumin; PBS: phosphate-buffered saline.

INTRODUCTION

Asthma is a chronic disease associating early acute bronchoconstriction with late bronchopulmonary hyperreactivity (BHR), airways eosinophilic inflammation, excessive mucus secretion and increased serum IgE titers [1-3]. The pathology of asthma involves Th2 CD4⁺ lymphocytes which produce excessive amounts of IL-4, IL-5 and IL-13. IL-5 is the major maturation factor for eosinophils in humans and in experimental animals, which regulates their growth, differentiation and activation [4-6], whereas IL-4 induces the

differentiation of Th0 into Th2 cells [7, 8] and stimulates the switch of B lymphocytes to the production of IgE [9, 10]. Elevated levels of IL-5 have been found in the blood and lung secretion from patients and correlate with the degree of eosinophilic lung inflammation [11, 12]. In addition, IL-5 titers are also increased, in the bronchoalveolar lavage fluid (BALF) and in the lungs, and correlate with the degree of BHR [11, 13, 14].

A number of studies using a mouse model of asthma have been conducted to clarify the role of IL-5 in the regulation of airways inflammation and BHR. The conclusions from these studies are controversial, since on one hand they provide strong evidence that IL-5 is the key regulator of eosinophilic inflammation and BHR [15-19], but on the other it has been reported that even though airways eosinophilia is reduced in mice when the IL-5 gene is disrupted [20] or when IL-5 is neutralized [21], BHR persists. Furthermore, the infiltration of eosinophils in the airways and lungs has been related to IL-5 [4], but their role in BHR has been either implicated [16, 17, 22] or discounted [20, 21, 23, 24]. In these studies, no distinction was made between eosinophil sequestration in the lungs and their recruitment to the alveolar compartment.

We have investigated the role of IL-5 in antigen-induced BHR and lung or BALF eosinophilia, using two different approaches. On one hand, the specific anti-IL-5 antibody TRFK-5 and its isotype GL113 were introduced directly into the airways, aiming at a selective local target, alone or associated with their systemic administration. As we show, this distinction is essential. On the other hand, in order to control the effectiveness of the antibody administration in the lungs depending on the route of administration, an *ex vivo* procedure was used, in which lung explants were allowed to proceed *in vitro* with the production and release of the cytokines, as happens *in vivo* in the presence or absence of the appropriate antibodies. Our results demonstrate that IL-5 is present in the alveolar, pulmonary and circulatory compartments after intra-pulmonary allergenic provocation of mice. Its neutralization in the BALF and in conditioned medium from lung explants with TRFK-5 administered i.n., was accompanied by the suppression of eosinophil recruitment to the BALF. Despite this, eosinophil lung sequestration was unchanged by i.n. TRFK-5 (alone or associated with i.v.) in BP2 mice and at the best, moderately reduced in BALB/c. Under those conditions, IL-5 being fully neutralized, BHR was not modified in BALB/c and only affected moderately in BP2, mice. Accordingly, neither IL-5 production in the lungs nor eosinophil migration to the BALF (which requires IL-5 in the alveolar

compartment) are causally related to BHR. Lung eosinophil sequestration involves IL-5-independent factors which may participate in BHR.

METHODS

Animals

Male BALB/c and BP2 mice aged 6 to 7 weeks were purchased from the Centre d'Elevage R. Janvier (Le Genest Saint-Isle, France). BP2 mice have been used in our laboratory [25], because under similar conditions of immunization and provocation they responded with an extensive long-lasting BHR, as compared to mediocre responses in BALB/c [15]. BP2 express optimal BHR after a single antigenic challenge, whereas BALB/c mice, which are extensively used in the literature, are less responsive. Because of these differences, a strict comparison was performed here. Animals had free access to conventional laboratory feed and water. Animals were handled in accordance with French State Council guidelines for the care and use of laboratory animals (Decree n° 87-849, October the 19th 1987), and was approved by the Institutional Animal Care and Use Committee.

Protocol for antigen immunization and challenge

Unless otherwise stated, BALB/c and BP2 mice were respectively immunized by s.c. injection of 10 µg and 1 µg ovalbumin (OVA, ICN Biomedicals, Aurora, OH, USA) dispersed in 1.6 mg Al(OH)₃ (Merck, Darmstadt, Germany) in 0.4 ml of 0.9% NaCl (saline) at Days 0 and 7. These doses were selected as optimal for BHR and IL-5 production. At Day 14, one week after the booster injection, immunized mice were challenged intranasally (i.n.) with 10 µg OVA in 50 µl of saline for about 10 s under anesthesia by i.v. injection of ketamine (Imalgene® 1,000, 35 mg/kg, Merial, Lyon, France). Control mice were challenged i.n. with the same volume of saline solution.

Evaluation of bronchopulmonary hyperreactivity

BHR was measured using a barometric plethysmography method. Unrestrained, conscious mice were placed in a whole body plethysmographic chamber (EMKA Technologies, Paris, France) which measured the respiratory waveforms. Animals were exposed to an aerosol of methacholine (Mch, Aldrich, Milwaukee,

WI, USA) for 20 s at 0.1 M for BALB/c mice and 0.05 M for BP2 mice, delivered by an nebulizer. These single doses were selected because they allowed expression of optimal BHR by inducing sub-maximal bronchoconstriction on sham-challenged animals, thus not requiring repeated, time-consuming aerosolisations of Mch. The index of airway obstruction was expressed as enhanced pause (Penh), in response to inhaled Mch [26]. Penh was calculated as: $\text{Penh} = [\text{Te (expiratory time)} / \text{Tr (relaxation time)}^{-1}] \times [\text{Pef (peak expiratory flow)} / \text{Pif (peak inspiratory flow)}]$. For the graphic representation, each value was expressed every minute and calculated from the average of three values of Penh recorded every 20 s. In order to simplify the interpretations, the area under the curve (AUC) was calculated for 10 min. The graphics in terms of AUC represent the quantitative expression of BHR.

Administration of TRFK-5

Monoclonal rat anti-murine IL-5 (TRFK-5) or the isotype control (GL113) were administered i.n., and/or intravenously (i.v.) at 15 mug, 30 min prior to allergenic challenge on Day 14, in BALB/c and BP2 mice. Rat anti-mouse IL-5 mAb was purified from ascitic fluids and purification was achieved by affinity chromatography on a protein G column (HiTrap affinity columns, Pharmacia Biotechnology, Uppsala, Sweden), after precipitation by ammonium sulfate. TRFK-5 antibody cross-reacts with IL-5 from different species, including mice [27], and humans [28].

Preparation of serum and bronchoalveolar lavage fluid

Mice were anesthetized by intraperitoneal injection of urethane (ethylcarbamate, 2 g/kg, Sigma, St Louis, MO, USA), and the abdominal cavity was opened. Blood samples were collected from the inferior vena cava and serum was obtained after centrifugation at 500 x g for 10 min and stored at -20° C until used. After exsanguination, the lungs were flushed, *via* the cannulated trachea, with 4 x 0.5 ml of a sterile PBS solution (10 mM phosphate buffer, pH 7.4). The total cell numbers were automatically counted (Coulter Counter ZM, Coultronics, Margency, France). Bronchoalveolar lavage fluid was cytocentrifuged for 10 min (Cytospin, Shandon, England). Slides were stained with a May Grünwald Giemsa-derived method (Diff Quick, Baxter Dade AG, Duedingen, Switzerland), and a total of 200 cells was counted for each sample by light microscopy, the percentage of each cell population being determined.

The BALF was centrifuged for 10 min at 1,850 x g, 4° C (Jouan, Saint-Herblain, France), and the supernatants were removed and stored at -20° C until used.

Evaluation of lung eosinophil peroxidase activity

To quantify the lung sequestration of eosinophils, eosinophil peroxidase (EPO) activity in the lung was evaluated in 96-well plates by a cytochemical enzyme assay [29]. Lungs were thoroughly washed through the airways and the pulmonary vasculature to remove cells, removed and homogenized (Potter-Elvehjem glass homogenizer, Thomas, Philadelphia, PA, USA) in 0.05 M Tris-HCl buffer pH 8 containing 0.1% Triton X-100 solution for 1 min. Lung homogenates were centrifuged for 15 min at 1,600 x g 4° C (Sigma, Bioblock Scientific 2K15, Illkirsh, France). EPO activity was measured in the supernatant, based on the oxidation of o-phenylenediamine (Sigma) by EPO in the presence of hydrogen peroxide. Incubations were carried out in duplicate, in the absence or presence of the peroxidase inhibitor 3-amino-1, 2,4-triazole (Sigma). Results were expressed as optical density (OD). This technique does not identify the exact location of the eosinophils in the lungs, between vessel walls or parenchyma but, because of the extensive washing, allows tissue sequestration to be distinguished from passage to the BALF.

Protocol for production of conditioned media from lung explants

Lungs from BALB/c and BP2 mice were removed 24 h after OVA challenge, flushed *via* the right heart with 5 ml of saline to remove blood. The remaining parenchyma was chopped randomly into 16 small pieces and distributed into 24-well plates. Four small pieces were used per well for each stimulant used. Lung explants were cultured for 24 h at 37° C and 5 % CO₂ in AIM V medium (Life Technologies, Grant Island, NY, USA) containing 1 % L-glutamine (Life Technologies) and gentamicin (10 mug/ml, Sigma). Then, supernatants from lung explants with medium alone or stimulated with 1 mug/ml of soluble anti-CD3 monoclonal antibody (mAb) (145-2C11), 1 mug/ml of lipopolysaccharide (LPS, *Escherichia coli* O55:B5, Sigma) or 10 mug/ml of OVA (ICN Biomedicals) were collected and stored at -20° C until used. Results are expressed in pg of cytokine produced per mg of lung tissue (dry weight) at the end of the assay.

Evaluation of cytokines

IL-4 in the BALF and in conditioned medium from lung explants in culture was evaluated by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtiter plates were coated with monoclonal rat anti-mouse IL-4 (BVD4-1D11, Perbio Sciences, Erembodegem-Aalst, Belgium), at 2 µg/ml diluted in 0.1 M carbonate buffer pH 8.2 and incubated overnight at 4° C. Plates were washed and blocked with PBS containing 1% of bovin serum albumin for 2 h. After washing, dilutions of recombinant murine IL-4 (15.6-1000 pg/ml) or samples were applied overnight at 4° C. Then, biotinylated rat anti-IL-4 antibody (BVD6-24G2, Perbio Sciences) was added at 0.5 µg/ml for 2 h at 4° C. Plates were washed and incubated with ExtrAvidin® peroxidase conjugate (1:2,000 to each well, Sigma) for 45 min at room temperature. Plates were developed with tetramethylbenzidine substrate (Kiregaard Perry Laboratories, Gaithersburg, MD, USA). The reaction was stopped with sulfuric acid 2N and the plates were read at 450 nm with an automatic microplate reader. The lower limit of detection of this assay is ~ 10 pg IL-4/ml sample.

IL-13 in conditioned medium from lung explants in culture was determined using a commercial ELISA (R&D Systems, Deutschland, Germany) according to the manufacturer's instructions. The lower limit of detection of this assay is ~ 20 pg IL-13/ml sample.

IL-5 in the BALF/serum and in conditioned medium from lung explants in culture was quantified by ELISA as described [30, 31]. The lower limit of detection of this assay is ~ 10 pg IL-5/ml sample.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to determine significance among the groups. If a significance variance was found, an unpaired Student's t-test was used to assess comparability between means. A value of $p \leq 0.05$ was considered to be significant.

RESULTS

Optimal doses for the immunization of BALB/c and BP2 mice

We first determined the optimal dose of OVA required to immunize and subsequently express allergy. Groups of BALB/c mice were immunized with 1, 10 or 100 mug of OVA in Al(OH)₃ twice, with a 7 day interval, and challenged i.n. with 10 mug OVA one week after the second immunization. Twenty-four hours after challenge, airways responsiveness to Mch, IL-4, IL-5 and eosinophil content in the BALF, and EPO lung content were evaluated. Control mice were immunized with Al(OH)₃ alone and challenged with OVA. BALB/c mice expressed BHR down to an immunizing dose of 10 mug of OVA (Figure 1A). These mice showed an intense BALF eosinophilia after challenge, immunization with 1 and 100 mug being less efficient (Figure 1C). The EPO activity in lung tissues was detected after challenge in the immunized mice with the same intensity, down to an immunizing dose of 10 mug OVA (Figure 1D). The amounts of IL-4 found in the BALF were also optimal for 10 mug (Figure 1B) and those of IL-5 in the BALF (Figure 1E) and in serum (Figure 1F) were similar for 10 and 100 mug, 1 mug being clearly less efficient. We concluded that 10 mug of OVA was the optimal immunizing dose of OVA in BALB/c mice.

In the case of BP2 mice, the procedure for immunization using 0.01, 0.1, 1, 10, and 100 mug OVA per mouse was otherwise similar to that applied to BALB/c. BP2 mice expressed BHR, as compared to control mice, down to an immunizing dose of 0.01 mug, 1 µg being optimal (Figure 2A). When a lower dose of OVA was used for immunization, the responses to Mch were similar to those of controls. Eosinophils were recruited to the BALF after OVA provocation, dose-dependently with respect to the immunizing amounts of OVA (Figure 2C). The EPO activity in lung tissues was detected after challenge in the immunized mice with the same intensity, similarly down to an immunizing dose of 0.1 mug OVA (Figure 2D). By contrast, IL-4 was produced at similar amounts down to 1 mug, the dose of 0.1 mug being less effective. No IL-4 was detected when 0.01 mug OVA was used (Figure 2B). Contrary to the other parameters, the production of IL-5 in the BALF was optimal at 1 mug of OVA (in some instances at 0.1 mug), lower and higher doses of OVA providing less IL-5 (Figure 2E). A similar trend was noted for IL-5 in serum (Figure 2F). We concluded that 1 mug of OVA was the optimal immunizing dose of OVA for BP2 mice, and that higher doses may lead to sub-optimal results, possibly because of negative feedback loops.

Effectiveness of TRFK-5 on IL-5 content in the BALF and serum depending on its route of administration, and its failure to modify IL-4 content in the BALF

To validate the effectiveness of TRFK-5 *in vivo*, the IL-5 content in the BALF and serum was evaluated. TRFK-5 or its isotype control, GL113 were administered at 15 µg i.v. and/or i.n. 30 min prior to the OVA challenge on Day 14. Administered before challenge, GL113 failed to suppress IL-5 in the BALF (Figures 3A and 3C) or in the serum (Figures 3B and 3D) in BALB/c and BP2 mice. In both strains, TRFK-5 i.n. neutralized IL-5 in the BALF, but not in the serum (Figures 3A, 3B, 3C and 3D). Conversely, IL-5 in the serum was inhibited only when TRFK-5 was administered i.v. (Figures 3B and 3D), under conditions where IL-5 in the BALF was not (Figures 3A and 3C). This indicated that IL-5 can be neutralized fully when delivered i.n. and i.v. to immunized and challenged BALB/c and BP2 mice. Irrespective of their route of administration, neither TRFK-5 nor GL113 altered the increased IL-4 content of the BALF in BALB/c and BP2 mice (Figures 4A and 4B).

Effects of TRFK-5 on airway responsiveness to methacholine and eosinophilia

The administration of GL113 i.n and i.v., prior to allergenic challenge, to BALB/c and BP2 mice did not prevent BHR (Figures 5A and 5D), nor eosinophilia (cell counts in the BALF and EPO of lung tissue) (Figures 5B and 5E). Irrespective of its route of administration, the airway responsiveness to Mch was unaffected by TRFK-5, at IL-5 neutralizing doses, in BALB/c (Figure 5A), under conditions where BHR was moderately reduced in BP2 mice. The administration of TRFK-5 i.v. failed to interfere with the recruitment of eosinophils into the BALF in both BALB/c and BP2 mice, whereas its administration i.n. or i.n. and i.v. significantly reduced the recruitment of eosinophils to the BALF in both BALB/c and BP2 (Figures 5B and 5E). Irrespective of the route of administration of TRFK-5, the accumulation of eosinophils in the lungs was unaffected in BP2 mice (Figure 5F). In BALB/c mice, the administration of i.n. TRFK-5 (alone or associated with i.v.) reduced EPO in the lungs, whereas the latter was not affected after i.v. administration in BALB/c mice (Figure 5C).

Interference of the anti-IL-5 antibody TRFK-5 with Th2 cytokine production by lung explants

Lungs from BALB/c and BP2 mice were removed 24 h after challenge with OVA and flushed *via* the right heart with 5 ml of saline to remove blood. The remaining parenchyma was chopped randomly into 16 small pieces, which were distributed in 24-well plates and cultured at 37° C. Four small pieces were used per well for

each applied stimulant. The lung dry weight, used to calculate cytokine production per mg of tissue, was determined after 24 h (peak time for cytokines). In some wells, the spontaneous production of cytokines was evaluated using lungs from control and from OVA-challenged mice (ongoing allergy). In other wells, the pan-T cell-stimulating agent anti-CD3 mAb (1 mug/ml) was added to activate whatever lymphocytes would be present (resident for control tissues or recruited to the lungs of mice after allergenic challenge, *i.e.*, during ongoing allergy). LPS (1 mug/ml) was applied to stimulate resident or newly recruited macrophages, dendritic cells or monocytes and finally OVA was added to other wells (10 mug/ml) to induce specific cytokine production. Medium alone served as a control.

As indicated, the i.v. and i.n. administration of TRFK-5 on Day 14, 30 min before OVA, suppressed IL-5 in the serum and in BALF (Figures 3A and 3C). In confirmation, and further validating the explant procedure, the production of IL-5 by the lung explant *ex vivo* was also suppressed by TRFK-5 administered i.v. and i.n. 48 h later (24 h in the animals plus 24 h in culture) in BALB/c and BP2 mice, both for ongoing allergy or after *in vitro* stimulation with anti-CD3 mAb or OVA (Figure 6A). Consistent with the fact that IL-5 in the BALF was only suppressed by i.n. TRFK-5, the i.v. route being inactive (Figure 3), the i.n. administration of TRFK-5 also suppressed the production of IL-5 by explant conditioned medium, whereas the i.v. route failed to do so (*Table 1*).

As seen in Figures 6B and 6C, lungs from OVA- immunized and -challenged BALB/c and BP2 mice treated before the allergenic challenge with GL113 i.n. and i.v., produced IL-4 and IL-13 whatever the stimulant. Explants from immunized mice alone failed to produce IL-4 or IL-13 after stimulation with LPS, as compared to medium alone, but secreted IL-4 and IL-13 after stimulation with the anti-CD3 mAb. TRFK-5 had no effect on IL-4 or IL-13 production. Overall, the i.n. or i.v. administration of TRFK-5 failed to interfere with the production of IL-4 and IL-13 (not shown).

DISCUSSION

It has been claimed that IL-5, the main growth factor for eosinophils [32, 33], plays a central role for in induction of eosinophilic airways inflammation and BHR in the mouse model of asthma [15-17]. Both BHR and lung eosinophil sequestration are abrogated in C57Bl/6 mice, in which the IL-5 gene is disrupted

[16], and is reconstituted by the adoptive transfer of IL-5-secreting CD4 T lymphocytes [18]. BHR is also reported in non-immunized mice over-expressing IL-5 in the lungs [22], even though we [34] have demonstrated that bronchoconstriction in response to i.v. 5-HT in IL-5 transgenic mice is similar to that of control CBA mice, irrespective of immunization. Anti-IL-5 antibodies are reported to suppress antigen-induced BALF eosinophilia, but the effects on BHR remain controversial. Thus, Eum *et al.* [15] and Hamelmann *et al.* [17, 19] claimed that IL-5 is essential for BHR, whereas Corry *et al.* [21], Nagai *et al.* [35] and Hogan *et al.* [20] argued against such a role. This led us to reinvestigate the role of IL-5 in murine BHR, by comparing two strains: BALB/c, which shows a Th2 bias, and BP2 [36], which expresses intense BHR after a single allergenic provocation [25]. Our approach was based on the recognition that eosinophil migration to the BALF is a partial, and sometimes distorted, reflex of lung eosinophil sequestration and that, under physiological conditions, the alveolar, the pulmonary and the vascular territories are strictly compartmentalized. Accordingly, eosinophils were counted in the BALF, and the EPO content was evaluated in the lungs, and analyzed separately. In addition, the anti-IL-5 mAb TRFK-5 and its isotype GL113, were administered i.n., in order to selectively reach the alveolar compartment, or i.v., in order to reach the different organs, excluding the alveoli (as indicated by failure of the mAb to suppress alveolar IL-5). In order to validate the negative results in terms of eosinophil sequestration and BHR, it was first essential to demonstrate that TRFK-5 suppressed its designed target, IL-5 *in vivo* and *ex vivo*, from the lung explants. The anti-IL-5 effectiveness of TRFK-5, if properly administered, was confirmed, since no IL-5 was detected in the BALF of OVA-challenged mice pre-treated i.n. Conversely, no IL-5 was found in blood, when TRFK-5 was injected i.v.. In fact, more IL-5 was found in the BALF when the mice were injected i.v. with TRFK-5 or in the blood, when it was instilled i.n. The *in vivo* results were confirmed *ex vivo*. The conditioned medium from surviving lung fragments was collected from control mice and from those challenged with OVA (ongoing allergy) and/or exposed *in vitro* to anti-CD3 mAbs, to target resident or newly recruited CD4⁺ lymphocytes, to LPS, to target monocytes and macrophages, and OVA itself. Conditioned medium contained cytokines consistently (see below), including IL-5, which was absent in the lungs collected from mice treated with TRFK-5 i.n. and i.v. or i.n, but not when the i.v. route alone was used. This means that the i.n. administration of TRFK-5 indeed eliminates IL-5 and validates the analysis of eosinophil sequestration and of BHR. Under these conditions, the administration of TRFK-5 by the i.n. or by both the i.n. and i.v. routes prior to allergenic challenge, inhibited eosinophil recruitment into the BALF in BALB/c and BP2 mice, whereas its i.v.

administration did not. The recruitment of eosinophils to the BALF may thus depend on the IL-5 actually present in the BALF and/or lungs, but is independent from its circulating titers. By contrast, in our experiments, lung eosinophil sequestration, in terms of EPO titers, was not affected by TRFK-5 in BP2 mice (even though IL-5 was suppressed), but was only reduced in BALB/c mice after i.n. TRFK-5 (alone or associated with i.v.), in which lung eosinophilia may thus be, at least in part, IL-5-dependent. This partial inhibition failed to affect BHR. The suppression of IL-5 by TRFK-5 in the BALF and in the serum, and in the lung explants was thus strictly dependent on its route of administration in both mice strains, the i.v. route failing to suppress that part of IL-5 which may be most relevant to lung diseases. This confirms the existence of distinct functional compartments for the distribution of the anti-IL-5 antibodies and possibly for IL-5 itself. Indeed, contrary to IL-4, IL-5 was found both in the BALF/lungs and in blood, following the i.n. challenge with OVA. This presence in blood is not due to leakage of IL-5 from the alveolar to the blood compartment, since the selective neutralization of IL-5 in the alveoli did not result in its reduction in blood, but even in its augmentation in the case of BP2 mice. It is thus more likely that IL-5 is generated in blood, when OVA leaks to vessels following its i.n. administration, during late exudation. Interestingly, Wang *et al.* [37] using a different approach in which the IL-5 gene was transferred, also noted compartmentalization. Nevertheless, the i.n. IL-5 gene transfer failed to reconstitute airways eosinophilia in IL-5-deficient mice, despite increased IL-5 titers in the BALF, confirming that local IL-5 is not sufficient for the development of antigen-induced airways eosinophilia. In contrast to our results, the intramuscular gene transfer reconstituted both IL-5 and eosinophils in the circulating blood following allergenic challenge. These results may be accounted for by the fact, that the IL-5-deficient mice used were C57BL/6, in which Foster *et al.* [16] have indeed shown a major role for IL-5.

Overall, our results underline the importance of the concept of lung compartmentalization, the efficacy of anti-IL-5 mAbs depending upon their route of administration. Most reported investigations on anti-IL-5 used a single route of administration to neutralize the IL-5 titers in mice [17, 19, 21, 38-42]. In these studies, the efficiency of the treatment was not evaluated in terms of IL-5 titers in the different compartments upon the administration of TRFK-5, but was based only on the interference with eosinophilia and/or BHR. In our study, we show that the effects of anti-IL-5 treatment on eosinophilia or BHR vary according to the route of administration and may differ according to the mice

strain. As a consequence, the negative results observed in the clinical studies with humanized antibodies [43] may result from an inappropriate choice of their route of administration.

Tomkinson *et al.* [40] demonstrated that the i.v. administration of TRFK-5 inhibited BHR and BALF and tissue eosinophilia in BALB/c mice. In contrast, we demonstrate that the i.v. administration of anti-IL-5 antibody to BALB/c mice suppresses IL-5 in the serum but not in the BALF, and fails to modify BHR and eosinophilia, indicating that antigen-induced BHR is dissociated from IL-5. These differences may result from the protocol of sensitization, or from antibody-dependent properties. Indeed, in our hands, the isotype GL113, administered at more than 30 mug/animal, showed consistent undesired inhibitory effects, that precluded the use of larger doses of TRFK-5, whereas Tomkinson *et al.* [40] used 50 mug/animal of control rat IgG.

In contrast to BALB/c mice, in which IL-5 was not needed for the development of BHR, TRFK-5 i.n., i.v. or both i.n. and i.v., attenuated but did not suppress BHR in BP2 mice, under conditions where the recruitment of eosinophils to the BALF was reduced but their accumulation in lung was unaffected. IL-5 may thus play a non-essential role in BP2 mice for the development of BHR. These results reflect differences in the underlying mechanism leading to the BHR in different strains, suggesting that IL-5-mediated BHR is genetically determined. Our data demonstrate that BHR is IL-5- and eosinophil-independent in BALB/c mice, but is more linked to this cytokine and to eosinophils in BP2 mice. Since the suppression of IL-5 attenuated, but failed to suppress BHR, other mechanisms may be involved.

Although a number of studies support the hypothesis that increased eosinophil numbers and/or their cytotoxic products in the murine airways determines BHR [16, 19, 44, 45], other studies have dissociated airway eosinophilia from BHR [20, 21, 23]. In contrast to IL-5-knockout C57BL/6 mice, in which there is a loss of BHR and of pulmonary eosinophilia [16], IL-5-knock-out BALB/c mice can develop BHR independently of eosinophils, supporting the above mentioned strain differences [20]. These differences may not explain all the discrepancies, since other studies using an anti-IL-5 in BALB/c or C57BL/6 mice have dissociated eosinophilia and BHR [20, 21, 23]. In both strains, and whatever their pertinence to the human disease, it is clear that the systemic compartment is not the route by which the anti-IL-5 antibody should be administered to inhibit lung

eosinophilia, and this may be so in humans. BHR and lung eosinophil sequestration are mostly maintained despite the neutralization of IL-5, and the production of IL-4 and IL-13 are not affected. These results suggest that IL-4 and/or IL-13 may be involved in the development of BHR and accumulation of eosinophils in the lung as mentioned in the literature. Further investigations with other mAbs against IL-13 and using IL-4/IL-13 traps should help clarifying the issue.

CONCLUSION

In conclusion, using two murine strains which express BHR and different markers of allergic airways inflammation, we demonstrated that IL-5 is generated in distinct compartments, alveolar, lung and blood, and that, in order to suppress it selectively and effectively, the anti-IL-5 mAb must be delivered by a route determined by the location of the target. It is essential to verify the efficacy of the anti-IL-5 antibody in the relevant compartments in order to assess the involvement of IL-5 in the immunopathogenesis of asthma, a concept which was not confirmed in this study.

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Figures



		



		



		



		



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Table 1
Suppression of IL-5 production by i.n. TRFK-5 administration but not by i.v. in lung explants in BALB/c mice

Lung explant cultures	Treatment			
	i.n.		i.v.	
	GL113	TRFK-5	GL113	TRFK-5
	IL-5 (pg/mg tissue)		IL-5 (pg/mg tissue)	
Stimulant				
Medium	2,781 ± 580	427 ± 102*	4,418 ± 436	4,829 ± 648
Anti-CD3	5,389 ± 667	58 ± 119*	4,218 ± 127	4,984 ± 768
LPS	2,603 ± 769	401 ± 93*	3,895 ± 554	3,612 ± 340
OVA	5,872 ± 1,077	387 ± 112*	3,916 ± 4,335	3,732 ± 595

At Day 14, OVA-immunized BALB/c mice were treated i.n. or i.v. with 15 µg TRFK-5 or GL113, 30 min prior to OVA challenge. At Day 15, mice were sacrificed and lung explants were cultured for 24 h in the presence of medium alone, anti-CD3 mAb (1 µg/ml), LPS (1 µg/ml) or OVA (10 µg/ml). IL-5 production in conditioned medium from lung explants in culture was quantified by ELISA. Values were normalized for dry tissue weight and expressed as pg per mg of tissue. Results shown as mean ± SEM (n = 4). *p < 0.05, TRFK-5-treated mice were compared to GL113-treated mice for each stimulant used.

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