

Low dose oral administration of cytokines for treatment of allergic asthma

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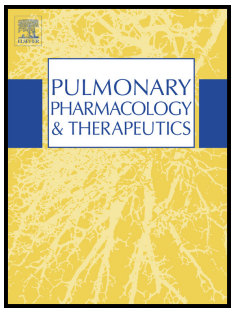
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Abstract

Many inflammatory diseases are characterized by an imbalance among lymphocyte populations, in particular Th1, Th2 and the recently described Th17 cells. The Th1/Th2 imbalance is linked to many factors, but certainly the role of cytokines is essential.

In Th2 diseases IL-4 expression is predominant, while Th1 pathologies are characterized by high expression of IFN-γ and IL-12. Though today the therapeutical proposal for many inflammatory diseases aims to re-establish normal levels of Th1/Th2 cytokines, the pharmacological use of cytokines, which are very active molecules, is limited by the possible collateral effects. Therefore, our study aims to determine, in a murine model of allergic asthma, the possible therapeutic activity of low dose cytokines solutions, mechanically activated. We found that oral administration of low doses IL-12 plus IFN-γ is able to solve the bronchial hyperresponsiveness condition of mice, establishing normal cytokine levels. The anti-asthma activity was confirmed by histological analysis of lungs and bronchoalveolar lavage fluid cell count. Serum ovalbumin-specific IgE was also significantly inhibited by treatment with low-dose activated cytokines solution. These findings may suggest a novel approach to diseases which involve a Th1/Th2 imbalance.

Keywords: allergy, cytokines, lung, mouse, Th1/Th2 Cells, IL-12, IFN-γ.

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1. INTRODUCTION

Asthma is a condition involving the respiratory system, in which the airways occasionally constrict, become inflamed and are coated with excessive amounts of mucus, often in response to one or more triggers. These episodes may be triggered by factors such as exposure to an environmental stimulant (or allergen) such as cold air, warm air, perfume, moist air, exercise or exertion, or emotional stress. In children, the most common triggers are viral illnesses such as those that cause the common cold. This airway narrowing causes symptoms such as wheezing, shortness of breath, chest tightness and coughing.

In particular, allergic asthma is a disease mediated by the expansion of the T helper 2 (Th2) subset of T cells, together with isotype switching of B cells, to generate IgE antibodies specific for common environmental allergens [1]. Other kinds of inflammatory diseases are characterized by a T-helper cells imbalance: these cells, after cellular or humoral stimulation, begin to divide and rise a clone. These effector Th cells are CD4+ and can be divided into Th1, Th2 or the latest Th17, with distinct cytokine secretion phenotypes eliciting unique functional characteristics for each type. Th1 cells secrete the cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-β, which allow these cells to be effective against intracellular infections by viruses and bacteria. Th2 cells secrete interleukin (IL)-4, -5, -10 and -13, which up-regulate antibody production and target parasitic organisms. On the contrary Th17 secrete IL-17, IL-17F, IL-6, IL-22 and TNF-α and appear to play an integral role in tissue inflammation and activation of neutrophils to combact extracellular bacteria [2].

The most abundant presence of one of these cell populations can be a warning of the event or progression of different diseases. In fact there are different kinds of disease such as multiple sclerosis, rheumatoid arthritis, diabetes Type I (insulin-dependent), Crohn's disease and psoriasis, with a greater presence of T helper 1 cells and cytokines such as IL-12 and IFN-γ [3]. Conversely, a characteristic allergic disease has a greater abundance of T helper 2 cells and their relative cytokines (IL-4, IL-5, and IL-13) [1]. These cytokine levels are the consequence of the imbalance between Th1 and 2 response and markers of the presence of the disease.

The precise mechanisms involved in preferential induction of Th1-type responses and down-regulation of Th2-type responses are still unknown. Anyway, it is now well established that selected cytokines from Th1- or Th2-type cells can down-regulate the expression of the opposite Th cell phenotype [4-6]. For example, IL-12 and IFN-γ directly induce T cells to differentiate into Th1 cells; it is exclusively the IFN-γ that also acts as an inhibitor of the Th2 pathway.

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On the basis of this knowledge, these cytokines have been used in several studies, in order to restore the balance between Th1/Th2 response, lost due to disease [7]. In particular, oral but not parenteral interleukin (IL)-12 redirects Th2-type responses to an oral vaccine without altering mucosal IgA responses [8] and suppresses anaphylactic reactions in a murine model of peanut hypersensivity [9]. Cytokines are though highly reactive molecules and it is known that their use can lead to side effects (e.g. autoimmune thyroiditis, rheumatoid arthritis, psoriasis, diabetes, multiple sclerosis). Therefore it would not be surprising to observe such disorders following the administration of cytokines [10] and it is necessary to watch out carefully for their use. For this reason it is useful to choose the most appropriate route of administration, to avoid possible side effects. Parenteral administration may deliver too much cytokine in the peripheral tissues with resultant adverse events, instead of delivering the cytokine only at the local disease site or to a portion of the immune system that might respond to the immunomodulatory effects of the cytokine. Furthermore, in animal and human experimental trials, systemic toxic effects associated with high doses of cytokines after parenteral administration are common complications, which have been demonstrated repeatedly under natural conditions of health or disease.

Another route, oral delivery, might offer a means of engaging the cytokine network, to foster beneficial effects in animals and humans [8, 11]. In addition oral administration of cytokines seems to give better results with respect to other routes [12].

Since different works have shown so far positive results using oral cytokine concentrations equal to 1000 and 500 ng [9], in this work we have tried to use lower doses of IL-12 and IFN-γ, observing their therapeutic effects in a Th2-type disease, such as the murine model of allergic asthma. The two cytokines were co-delivered orally, enhancing their individual effects. The significance of IL-12- plus IFN-γ-induced Th1-type responses, for regulation of lung and consequent systemic immunity, is here discussed.

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2. METHODS

2.1. Preparation of cytokines solutions

All dilutions and preparations of cytokines were prepared starting from a concentrated solution (1µg/ml) of recombinant murine IL-12 (Sigma-Aldrich, Saint Louis, MO) and of recombinant murine IFN-γ (Sigma-Aldrich). Different dilutions were performed using a 30% hydro-alcoholic solution as diluent. For each administration, a total volume of 200 µl was administered to each animal, ones a day. The different cytokines dosages tested were: 500 ng/dose, 100 ng/dose, 1 ng/dose, 10 pg/dose, 100 fg/dose, 1 fg/dose, 10^{-2} fg/dose and 10^{-4} fg/dose. Based on previous knowledge on activated blends [13, 14], the low-dose cytokine solutions were prepared in 2 different formulations: an activated solution and a non-activated solution. The non-activated solutions were prepared by simple serial dilutions 1:100, diluting the concentrated solution into the solvent. The activated solutions, following serial dilutions 1:100, underwent a shaking process: in detail, the applied shaking procedure is characterized by the following parameters: vertical shaking; 10 centimetres motion range; shaking speed corresponding to 100 oscillations in 10 seconds.

2.2. Experimental asthma model induction

Male FVB mice, which are highly susceptible to asthma-like airway hyperresponsiveness, were purchased from Charles River, Italy (Head Office Wilmington, MA). Mice were housed under specific pathogen-free conditions, maintained at constant temperature and humidity, with food and water given ad libitum, and used at 8-10 weeks of age. Animal experimentation is authorised by the Government in the Decree Law 116/92, according to the European Economical Community (CEE) guidelines number 609/86.

The experimental asthmatic state was induced in animals referring to previously described models [15-18]. In brief, mice (n=5/group) were immunized by i.p. injection of 100 µg of ovalbumin (OVA grade II; Sigma-Aldrich) in association with 500 µg of aluminium hydroxide (Alum, Sigma-Aldrich) (T=0, Figure 1). One week after the first immunization, the same treatment was repeated (T=7, Figure 1). Two weeks after the first immunization, animals were challenged for 25 min by aerosol administration with a solution containing 5% (wt/vol) OVA in PBS pH 7.4 each day, for 5 consecutive days (T=13-17, Figure 1); oral cytokine treatment started on the following day (T=18, Figure 1). On the 10^{th} day of oral cytokine treatment (T=27, Figure 1), mice received an additional challenge with i.p. injection of 100 µg of OVA in association with 500 µg of Alum. Again, on the 13^{th} day of oral cytokine treatment, mice were challenged for 25 min by aerosol administration with a solution containing 5% (wt/vol) OVA in PBS pH 7.4 (T=30, Figure 1).

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2.3. Animals treatment with cytokines solutions

Mice were divided in different groups for the different treatments, 5 mice per group. Following induction of asthma, mice were treated with the different cytokine solutions, ones a day, for 20 days totally (T18-T38, Figure 1), by oral route, using a stomach tube. The first treatment groups of mice received IL-12 alone in the following dosages: 500 ng/dose, 100 ng/dose, 1 ng/dose, 10 pg/dose, 100 fg/dose, 1 fg/dose, 10^{-2} fg/dose and 10^{-4} fg/dose. The 1 ng/die, 10 pg/die, 100 fg/die, 1 fg/die, 10^{-2} fg/die and 10^{-4} fg/die dosages were administered to mice in the two different formulations previously reported: an activated solution and a non-activated solution. Other treatment groups received the previously described low doses of IL-12 (1 ng/die, 10 pg/die, 100 fg/die, 1 fg/die, 10^{-2} fg/die and 10^{-4} fg/die), in association with equivalent dosages of IFN-γ, both in the activated and non-activated formulations, co-delivered by oral route. On days 4^{th}, 7^{th}, 12^{th}, 16^{th} and 20^{th} of oral cytokine treatment (T21, T24, T29, T33 and T38, respectively, Figure 1), serum samples were collected from the retro-orbital sinus of mice. Mice were sacrificed on the 20^{th} day of oral cytokine treatment (T38, Figure 1) and broncho-alveolar lavage was performed. Some treated animals were not sacrificed at the end of treatment, but were maintained alive for an additional week (T45, Figure 1), in order to evaluate the variations in cytokine levels when the treatment with cytokines has been stopped.

2.4. Broncho-alveolar lavage

Broncho-alveolar lavage was performed by instilling 1 mL saline into the lungs through a tracheal cannula and then gently aspirating the fluid. This lavage was repeated three times. The recovered fluid was filtered through a double layer of sterile gauze to remove mucus. The resulting broncho-alveolar lavage fluid (BALF) was centrifuged (1000 rpm, 4º C, 10 min) and supernatants were collected; cells were fixed in 1% formalin with the addition of 2% sucrose and collected on glass slides by cytocentrifugation. Infiltrated cells identification and counts were performed on Giemsa-stained cytospined slides of the cells.

2.5. Hematoxylin–eosin staining

The lungs collected from different animal groups were fixed by inflation with 10% paraformaldehyde solution and embedded in paraffin. Sections were cut at 4 μm thickness, deparaffinized, rehydrated, stained with hematoxylin and eosin and mounted in Entellan® (Merck). Slides were observed under a Nikon Eclipse 80i microscope equipped with a digital Nikon DS-L1 camera (Nikon).

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2.6. Masson-trichrome staining

To estimate the degrees of fibrosis, Masson-trichrome staining was performed. The 4 μm thickness slides were deparaffinized and rehydrated in the distilled water. The sections were first stained with Weigert iron hematoxylin, followed by Biebrich scarlet-acid fuchsin staining; after differentiation in 1% acetic water for 1 min, slides were treated with a solution containing phosphowolframic acid and Orange G in distilled water. Finally, samples were stained with Fast Green FCF solution in 1% acetic water, dehydrated and cleared through graded ethyl alcohol.

2.7. ELISA

The quantitative determination of total IL-12 (IL-12p70 and IL-12p40), IFN-γ, IL-4 and IL-5 concentration in diluted serum samples and in BALFs was performed using commercially available enzyme-linked immunosorbent assay kits purchased from Pierce Biotechnology (Rockford, IL), according to manufacturers' instructions. Mouse OVA-IgE, IL-13 and IL-17 ELISA kits were from MD Biosciences (Zürich, Switzerland), Bender MedSystem GmbH (Vienna, Austria) and BioLegend (San Diego, CA), respectively. The enzymatic reaction was detected in an automatic microplate photometer (Multiskan Ascent, Thermo Electron Corporation, Vantaa, Finland). Concentrations in the unknown samples were determined by interpolation into a standard curve, developed with known amounts of recombinant peptides.

2.8. In vitro treatment of splenocytes

Murine spleens were removed aseptically from untreated mice and placed into Petri dishes with 4 ml cold RPMI medium 1640 (Gibco Invitrogen Corp., Auckland, NZ). Spleens were gently broken apart using a 2-ml syringe to produce a single-cell suspension and centrifuged at 1500 rpm for 5 min at 4° C. The cell pellets were disrupted by light agitation, erythrocytes were lysed hypotonically, and splenocytes were resuspended in RPMI medium 1640 containing 10% FBS (Gibco Invitrogen Corp.) and 10 ml/l of penicillin and streptomycin (Sigma-Aldrich). Splenocytes were plated in a 24-wells plate (100,000 cells/well) and treated *in vitro* with low doses of both activated and non-activated IL-12 and IFN-γ for 24 hours. The concentration of cytokines in the cell medium was analogous to the concentration in the solutions administered to mice; in particular, the activated and non-activated solutions of mixed IL-12 and IFN-γ tested were the following: 1 pg/ml (corresponding to the 100 fg/die dosage to mice), 10 fg/ml (corresponding to the 1 fg/die dosage to mice), and 0.1 fg/ml (corresponding to the 10^{-2} fg/die dosage to mice). At the end of treatment, cells supernatants were collected and analysed for cytokines content by ELISA.

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2.9. In vitro treatment of CD11c+ dendritic cells

Murine splenocytes were isolated from untreated mice as previously described. The single-cell suspension was washed with PBS containing 0.5% FBS, cells were then counted and, following pre-incubation with anti-Fc receptor antibody (Miltenyi Biotec S.r.l., Calderara di Reno, Italy), incubated at the appropriate ratio with MACS CD11c microbeads (Miltenyi Biotec S.r.l.) for 15 min at 4°C. After being washed again with PBS containing 0.5% FBS, cells were separated by passing the antibody-coated cell suspension over a VS+ column on a SuperMACS magnetic cell separator (all from Miltenyi Biotec S.r.l.). CD11c-positive cells were collected by removing the column from the magnetic field and then flushing it with PBS-0.5% FBS. Macrophages were removed from the CD11c+ cell population by plastic adherence incubation overnight at 37°C in a 5% CO_2 atmosphere. Thereafter, cells were washed with RPMI medium and used for the following *in vitro* treatment. In brief, CD11c+ dendritic cells were resuspended in RPMI medium 1640 containing 10% FBS (Gibco Invitrogen Corp.) and 10 ml/l of penicillin and streptomycin (Sigma-Aldrich), plated in a 24-wells plate (100,000 cells/well) and treated *in vitro* with low doses of both activated and non-activated IL-12 and IFN-γ for 24 hours. The concentration of cytokines in the cell medium was analogous to the concentration in the solutions administered to mice; in particular, the activated and non-activated solutions of mixed IL-12 and IFN-γ tested were the following: 1 pg/ml (corresponding to the 100 fg/die dosage to mice), 10 fg/ml (corresponding to the 1 fg/die dosage to mice), and 0.1 fg/ml (corresponding to the 10^{-2} fg/die dosage to mice). At the end of treatment, cells supernatants were collected and analysed for cytokines content by ELISA.

2.10. Statistical analysis

Student's *t*-test (paired two-tailed) and GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA) were used for comparisons between groups. *P* values less than 0.05 were considered significant.

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3. RESULTS

3.1. Oral administration of a pharmacological dose of IL-12 is efficacious in an experimental murine model of asthma

IL-12 is a heterodimeric cytokine produced by antigen-presenting cells, which promotes differentiation of Th1 cells and IFN-γ production, while inhibiting the differentiation of Th0 cells into IL-4-secreting Th2 cells [19]. It was recently shown [9] that oral administration of 500 ng/dose of recombinant mouse IL-12 complexed with liposomes is able to suppress anaphylactic reactions in a murine model of peanut hypersensitivity. Given this beneficial activity of oral IL-12 in food allergy, we aimed to assess if the same pharmacological dose of IL-12 might be a satisfactory treatment against allergic asthma.

After induction of an asthma-model condition, mice (n=10) were orally treated daily with 500 ng/dose recombinant mouse IL-12, for 20 days totally, while a control group (n=5) received the IL-12 vehicle alone (sham-treated group). OVA-untreated mice (control group, n=5) served as reference for the basal non-pathological condition. The severity of experimental asthmatic state in mice was monitored throughout and at the end of treatment period, by evaluation of levels of different cytokines in serum samples and in BALF. As shown in Figure 2, sham-treated mice exhibited highly increased serum levels of IL-4 and IL-5, the typical cytokines expressed by Th2 cells, which remained elevated throughout the whole treatment period, while no increase was observed in the Th1-related cytokine levels, specifically IL-12 and IFN-γ. The same imbalance in cytokine levels was observed in BALF of the same animals (Fig. 2), together with a significantly greater number of eosinophils, neutrophils and mononuclear cells than in the controls (Table 1), a clear index of a serious bronchial hyperreactive condition [20]. As regards mice treated with 500 ng/dose IL-12, they presented a completely different cytokine expression pattern, which revealed a complete recovering from the bronchial hyperreactive state with a predominance of Th1-related cytokines (Fig. 2): in fact, starting from the first days of treatment, the serum levels of IL-4 and IL-5 gradually decreased, with a concomitant increase of IL-12 and IFN-γ; moreover, BALF collected at the end of treatment showed a complete absence of eosinophilic, neutrophilic and mononuclear cells infiltrate (Table 1), high levels of IL-12 and IFN-γ, and low levels of IL-4 and IL-5 (Fig. 2). Five of the IL-12-treated animals were not sacrificed at the end of treatment, but were maintained alive for an additional week, in order to evaluate the variations in cytokine levels when the treatment with IL-12 was stopped (T45 follow-up in Fig. 1): we found that 7 days after the end of treatment the serum cytokine levels of these mice did not return to basal levels, but maintained IL-12 and IFN-γ concentrations higher than to the ones observed in the control group (Fig. 2). This

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seemed an important issue for us, since the persistence of high levels of IL-12 and IFN-γ after the end of treatment might be dangerous, bringing to the development of one of the pathologies linked to a Th1 imbalance, such as Crohn's disease [21]. This observation brought us to think about reducing the amount of oral IL-12 administered to mice.

3.2. Oral administration of low doses of activated IL-12 maintains efficacy in an experimental murine model of asthma

In recent years the development of new therapeutical approaches based on the administration of cytokines has been influenced by the possible development of collateral effects, since these molecules exert a very strong biological activity. Therefore, the possibility to obtain good therapeutical results by the use of low doses of cytokines should be the topic of new cytokine-based therapies. With this aim, since the daily dose of 500 ng/mouse recombinant mouse IL-12 proved to be efficacious against the experimental asthmatic state, but induced the maintenance of increased cytokine levels also after the end of treatment, we have tried to progressively reduce the amount of orally-administered IL-12, evaluating the recovery of animals from the bronchial hyperreactive state, as previously described. A first group of asthma-model mice (n=10) was daily treated with 100 ng/mouse IL-12, for 20 days totally: as shown by Fig. 3, all mice recovered from asthma, as shown by their serum levels on 7^{th} day of treatment (T24) and BALF cytokine levels, demonstrating that 100 ng/die IL-12 was a dosage as efficacious as the previously tested 500 ng/die. Moreover, BALF collected from these animals at the end of treatment showed a complete absence of eosinophilic, neutrophilic and mononuclear cells infiltrate (Table 1). Again, five of the 100 ng/mouse IL-12-treated animals were not sacrificed at the end of treatment, but were maintained alive for an additional week, in order to evaluate the variations in cytokine levels when the treatment with IL-12 was stopped: also in this case we found that 7 days after the end of treatment (T45) the serum cytokine levels of these mice did not return to basal levels, but remained a bit higher than the ones observed in the control group (Fig. 3).

Given these results, we have treated other groups of mouse asthma model with lower IL-12 doses, starting from serial 1:100 dilutions of the tested 100 ng/die dose: thus the different groups of animals (n=5/group) received 1 ng/die, 10 pg/die, 100 fg/die, 1 fg/die, 10^{-2} fg/die and 10^{-4} fg/die respectively. Each one of these dosages was administered to mice in two different formulations: an activated solution and a non-activated solution. As shown by Fig. 4, among non-activated solutions the dosage of 1 ng/die maintained a good efficacy against the experimental asthmatic state of mice, even though the cytokine levels revealed in BALFs at the end of treatment were not equal to the ones of control mice, while lower dosages proved not to be effective in experimental asthma

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treatment, as shown by serum and BALF cytokine levels. Very different results were obtained with activated solutions: in fact a good therapeutic activity was still maintained reducing the dose administered to 10 pg/die, with a complete recovering from the experimental asthmatic state. Indeed Fig. 5 demonstrates that mice treated with 10 pg/die of activated IL-12 show reduced levels of IL-4 and IL-5 and increased levels of IFN-γ and IL-12, in both sera and BALF, after 20 days of treatment, even though also in this case the cytokine levels revealed at the end of treatment were not equal to the ones of control mice. The lower dosages of activated IL-12 proved not to be effective against the induced allergic asthma. All the results obtained evaluating cytokine levels were further confirmed by eosinophils, neutrophils and mononuclear cells counts in the BALFs collected after sacrifice of animals: in fact only a very slight cellular infiltrate was present in lungs of mice receiving 1 ng/die non-activated IL-12 solution and 10 pg/die activated IL-12 solution, while groups of animals receiving lower dosages showed a noticeable presence of inflammatory cells in the broncho-alveolar space (Table 1). Once again, five of the 1 ng/die non-activated and 10 pg/die activated IL-12 solution-treated animals were not sacrificed at the end of treatment, but were maintained alive for an additional week: in this case, we found that 7 days after the end of treatment (T45) the serum cytokine levels of these mice returned to basal levels, comparable to the ones observed in the control group (Fig. 6).

3.3. Oral association of activated IL-12 and IFN-γ is still efficacious at dosage of 1 fg/die/mouse

We have shown that an activated solution of IL-12 orally administered to experimental asthmatic mice is efficacious against all the typical signs of the hyperreactive status until the dosage of 10 pg/die/mouse. It is known that IL-12 represents a key component in the network of cytokines, with complex positive and negative feedback regulatory mechanisms that are able to determine the type of Th immune response at the very early stages of antigenic challenge; in particular IL-12 production by accessory cells is a requirement for optimal production of IFN-γ by lymphocytes [19]. Thus we have hypothesised that the positive effects we have obtained in experimental asthmatic mice treated with low doses of IL-12 might be linked to both the activity of IL-12 on its own and to its ability to induce IFN-γ production by NK and T cells [19], as suggested by the high levels of both these cytokines which were found in sera and BALFs of IL-12 treated animals. Thus we assumed that it was possible that the simultaneous oral administration of the low dosages of IL-12 previously tested together with low dosages of IFN-γ might bring to an amelioration of the anti-inflammatory activity in lung, since low doses of IFN-γ may directly act recruiting further T cells in the inflamed region and helping in re-establishing a balanced Th1/Th2 status [1]. Therefore, we

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have tested again the previously described low doses of IL-12 (1 ng/die, 10 pg/die, 100 fg/die, 1 fg/die, 10^{-2} fg/die and 10^{-4} fg/die), in association with equivalent dosages of IFN-γ, co-delivered by oral route. Again, the associations of the 2 cytokines were tested both in a non-activated and in an activated formulation. The oral treatment of mice was performed for 20 days, as described above. As shown by Figures 7 and 8, the co-delivery of the 2 cytokines markedly ameliorated the results obtained on murine asthma model, if compared with solutions containing IL-12 alone; in particular the activated associations of IL-12 and IFN-γ proved to be effective against experimental asthma also at the very low dosage of 1 fg/die/mouse, bringing to a complete recovering from the bronchial hyperreactive state of mice, as assessed by cytokines dosage by ELISA and by inflammatory cells counts (Table 2), which were comparable at all to those of the control group. The 10^{-2} fg/die and 10^{-4} fg/die activated associations doses were not efficacious against experimental asthma (Fig. 8). Further, histological analysis of lung parenchyma by hematoxylin-eosin staining showed that lung sections from mice receiving 1 fg/die/mouse activated association of IL-12 and IFN-γ had no infiltration of inflammatory cells; in contrast, patchy cellular infiltration of the airways and lung parenchyma, with abundant eosinophils and enlarged macrophages, was seen in lung sections from experimental asthmatic animals (Fig. 9). Masson-trichrome staining was used to reveal peribronchial fibres. In sham-treated mice, the collagen fibres around the airways were significantly accumulated compared with the control animals; oral treatment with activated association of IL-12 and IFN-γ reverted the hyperreactive state, showing no significant differences in peribronchial collagen fibres respect to the control group (Fig. 9).

As regards the non-activated IL-12 and IFN-γ associations, only the 1 ng/die dosage maintained a good efficacy against the induced bronchial hyperreactive state, while lower doses had a slight (10 pg/die) or null (100 fg/die and lower doses) effect on experimental asthmatic mice (Fig. 7). Thus all these experiments show that the association of IL-12 and IFN-γ in an activated formulation, at the dosage of 1 fg/die/mouse, is the lowest oral dose which, if administered for 20 days to experimental asthmatic mice, is able to bring to the complete recovery from the bronchial hyperreactive state.

3.4. Further experiments on the effects of the 1 fg/die/mouse dosage of activated IL-12 and IFN-γ solution

Because Th2 cytokines promote airway inflammation in asthma through increased IgE levels, we investigated the expression of antiovalbumin IgE in serum of mice treated with the 1 fg/die/mouse dosage of activated IL-12 and IFN-γ solution, in comparison to the untreated murine asthma model and to control animals, at two different time-points (T18 and T38). Cytokine treatment was associated with a trend toward decreased production of antiovalbumin IgE: in fact, the levels of

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serum antiovalbumin IgE were found to be significantly increased in asthmatic mice compared with those of control mice, while administration of activated IL-12 and IFN-γ significantly decreased the levels of serum IgE at T38 (Fig. 10).

IL-13, together with IL-4, plays a critical role in the pathogenesis of asthma. Therefore, we evaluated the levels of this cytokine in the BALF of mice treated with the 1 fg/die/mouse dosage of activated IL-12 and IFN-γ solution, in comparison to the untreated murine asthma model and to control animals. Interestingly, oral administration of activated cytokines regulated the cytokine imbalance in local airways with decreased IL-13 levels, comparable to basal ones, with respect to the sham-treated group (Fig. 10).

Apart from Th1 and Th2, other T cell lymphocyte populations, notably Th17 cells, have been shown to play a role, at least in mouse models of allergic inflammation. Therefore, we evaluated the presence of this cell population in mice treated with the 1 fg/die/mouse dosage of activated IL-12 and IFN-γ solution, in comparison to the untreated murine asthma model and to control animals. As compared with control group, the expression of IL-17 protein in serum of asthmatic group was significantly increased, while its expression in cytokines-treated group was comparable to control group (Fig. 10).

3.5. *In vitro* treatment of splenocytes with low doses of activated IL-12 and IFN-γ induces high IL-12 and IFN-γ secretion

All our *in vivo* experiments with low doses of oral cytokines in the murine asthma model have shown that the recovery from the bronchial hyperreactive state was linked to the induction of high serum and BALF levels of Th1 cytokines IL-12 and IFN-γ. In order to determine how low doses of oral IL-12 and IFN-γ affected systemic Th1/Th2 responses, we measured cytokine levels in spleen cell supernatants following *in vitro* treatment of splenocytes with low doses of both activated and non-activated IL-12 and IFN-γ for 24 hours. The concentration of cytokines in the cell medium was analogous to the concentration in the solutions administered to mice; in particular, the activated and non-activated solutions of mixed IL-12 and IFN-γ tested were the following: 1 pg/ml (corresponding to the 100 fg/die dosage to mice), 10 fg/ml (corresponding to the 1 fg/die dosage to mice), and 0.1 fg/ml (corresponding to the 10^{-2} fg/die dosage to mice). Evaluation of cytokine levels in splenocyte supernatants by ELISA revealed that the very low doses of activated cytokines used were able to stimulate spleen cells to secrete significant levels of IL-12 and IFN-γ, as shown by Fig. 11, while maintaining null concentrations of IL-4 and IL-5 (data not shown). In particular, the 10 fg/ml (corresponding to the 1 fg/die dosage to mice) concentration of cytokines was the lowest concentration which induced a significant Th1 cytokines production, analogously to the results

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previously obtained *in vivo* with the 1 fg/die dosage to animals. These results obtained with activated cytokines were not achieved by the use of non-activated solutions, as previously observed on mice (data not shown).

The same *in vitro* treatment was analogously repeated on CD11c+ dendritic cells only, isolated from whole splenocytes by magnetic cell sorting. In this case the 1 pg/ml and the 10 fg/ml concentrations of activated IL-12 plus IFN-γ induced a marked increase of IL-12 secretion in the cell supernatant (Fig. 12), demonstrating that these APCs were responsible for the high IL-12 levels which were found in mice treated with the oral association of activated IL-12 and IFN-γ at the dosage of 1 fg/die/mouse.

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4. DISCUSSION

In this work we have demonstrated that very low dosages of activated solutions of IL-12 and IFN-γ, co-delivered by oral route to experimental asthmatic mice, are able to revert their pathological condition, restoring a normal balance between Th1 and Th2 cytokines and bringing to a healthy state. Systemic administration is not an efficient way to deliver cytokines to specific inductive sites, while administration of cytokines via the oral route offers an exciting alternative to systemic application, for ease of dispensation. Increasing evidence suggests that oral administration of certain cytokines is not only safe and effective, but also avoids the deleterious consequences of systemic administration, retaining sufficient biological activity to effect immunomodulatory functions beyond the local mucosa [22].

Differentiated mouse CD4+ T cells produce a restricted set of cytokines, allowing their subdivision into three discrete populations: Th1, characterized by the secretion of IFN-γ; Th2, which selectively produce IL-4, IL-5 and IL-10; Th17, linked to the pro-inflammatory IL-17 cytokine family [23]. A similar distinction also applies to human T cells. The development of antigen-specific CD4+ cells into polarized Th1, Th2 and Th17 subsets is influenced by several factors, including the cytokine milieu during the initial phase of the immune response [24]. Among cytokines, decisive roles are played by IL-12 and IL-4, driving T cell responses towards the Th1 or Th2 phenotype, respectively [25, 26]. IL-12, produced by macrophages and DCs, represents a key component in the network of cytokines, with complex positive and negative feedback regulatory mechanisms that are able to determine the type of Th immune response at very early stages of antigenic challenge [19]. One could envision two IL-12 effects, e.g., the initial promotion of Th1-type responses from naïve T cell precursors, or a reversal of an established Th2-type response by initiation of Th1-type responses [27, 28], and evidence for both pathways has been put forth. Our work is based on this second effect of IL-12, since we have shown that co-delivery of very low doses of activated IL-12 and IFN-γ to mice with the typical Th2 imbalance found in asthma initiates a Th1-type response, re-establishing a non-pathological condition, as shown by the different parameters analysed: histological analysis, BALF cell counts and cytokine or IgE levels. Thus, if the use of IL-12 to direct Th1-type responses has well-known important implications for therapy [29, 30], the novelty of our work resides in the association of the two Th1-typical cytokines, which are used at a very low dose and prepared as an activated solution.

As regards the co-delivery of the two cytokines, after preliminary studies based on the use of low doses activated IL-12 alone, we have preferred an association of IL-12 plus IFN-γ low-dose activated solutions. In fact, it is well known that IL-12 produced by APCs is a potent stimulator of

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IFN-γ production by NK and T cells [25, 31, 32] and most of IL-12 effects are mediated through IFN-γ synthesis [25]. The link between IL-12 and IFN-γ has been studied extensively, and several lines of evidence support the view that these cytokines positively regulate each other. IFN-γ in vivo depends on IL-12 [33-35], and the ability of IL-12 to protect mice from parasitic infections is mediated by IFN-γ [36]. In addition, IL-12 secretion by macrophages is enhanced by the priming of the IL-12p40 gene by IFN-γ [37]. Moreover, while IFN-γ, IL-12 and type I IFNs directly induce T cells to differentiate into Th1 cells, it is exclusively the IFN-γ from APCs and natural killer cells that also acts as an inhibitor of the Th2 pathway by preventing the Th2 cell proliferation [3, 38].
A protective role of IFN-γ has been previously shown in murine models of asthma in which mucosal IFN-γ gene transfer and nebulised IFN-γ application inhibited eosinophilic inflammation [39, 40]. Indirect evidence for a protective role of IFN-γ has also been demonstrated in a human study, where allergen immunotherapy increased IFN-γ production by circulating T lymphocytes in patients, with clinical benefit [41]. Because the Th1 cytokine IFN-γ cross-regulates Th2 cells in some systems [5], IFN-γ is thought to be critically involved in down-modulating Th2 cell-driven asthma. Thus, our initial hypothesis for the co-delivery was based on all these literature data, since it seemed to us that an association of the 2 cytokines might amplify the positive effects we had yet observed with low-dose of activated IL-12 alone. The results we have obtained confirmed our hypothesis: in fact, if low-dose activated solutions of IL-12 alone were efficacious against asthma until the dosage of 10 pg/die, the solutions containing both low-dose activated IL-12 plus IFN-γ were still effective at the dosage of 1 fg/die.
Regarding the attempt to develop very low-dose cytokine administration, this choice is in accordance with data of literature, suggesting that low doses of cytokines are adequate in many different models [22]; the low-dose effects in these models strongly suggest the induction of a secondary mode of action, perhaps the induction of cytokine secretion in cells other than the ones directly stimulated by the administered cytokines. For example, in vivo experiments of low-dose interferon-α treatment have been described in many animal species [11]; such a treatment was shown to induce dramatic clinical amelioration in models of both infectious and chronic inflammatory diseases [42]. Low-dose, oral IFN-α treatment was also shown to exert an anti-inflammatory activity in standard-bred racehorses affected by inflammatory airway disease; in 8 to 15 days a significant reduction of neutrophils, macrophages and lymphocytes was observed in bronchoalveolar lavage fluid in IFN-α-treated animals, as opposed to controls [43]. Our results show that very low dosages (until 1 fg/die) of activated IL-12 plus IFN-γ cytokine solutions maintain a strong efficacy against the asthmatic state of our murine model; moreover we can

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assume that the high efficacy is not accompanied by the collateral effects present with higher cytokine dosages. For example, we have observed that with higher doses (100 ng/die) of administered cytokines, the serum levels of Th1 cytokines remained high also 7 days after the end of treatment: this aspect might be dangerous [44, 45], since the maintenance of serum Th1 cytokine levels higher than the basal ones might bring to deleterious effects, with a Th1 imbalance opposed to the asthmatic Th2-driven condition. On the contrary, animals treated with lower cytokines doses reported basal serum Th1 cytokine levels 7 days after treatment end: thus, low-dose administration shows both efficacy and safety of use.

The most intriguing aspect of our work regards the difference of activity we have noted between activated and not-activated cytokine solutions, since the first ones proved to be greatly more efficacious than the not-activated counterparts. The use of activated blends is well-known in different fields, including pharmaceutical technology, though the mechanisms underlying their increased efficacy is not well-known at all. Here we report only a mere observation of the highly increased efficacy of activated versus not-activated low-dose cytokines solutions, which allows to obtain a high therapeutical activity with very low dosages, though we still do not have a clear explanation of this phenomenon.

The low-dose effects of activated cytokine solutions in our model of murine asthma strongly suggest the induction of a secondary cytokine secretion in different immune cells. Trying to further confirm this hypothesis, we have measured cytokine levels in spleen cell supernatants following *in vitro* treatment of splenocytes with low doses of both activated and not-activated IL-12 and IFN-γ. It was previously reported that addition of IL-12 (10 ng/ml) to splenocyte cultures resulted in high levels of IFN-γ [7]. Our results demonstrate that lower doses (10 fg/ml) are sufficient for the induction of Th1 cytokines by splenocytes; in particular, the splenocytes mainly involved in IL-12 and IFN-γ productions might be T cells and APCs. To date, few studies have addressed the role of APCs in the induction of mucosal immune responses. A major role for APCs is their contribution to the cytokine environment for growth and development of Th1- or Th2-type cells. Isolation of CD11c+ dendritic cells only showed that these APCs were the main responsible for the high IL-12 levels found in mice treated with low-dose activated co-delivery of IL-12 plus IFN-γ.

Though further study is needed to establish the regulatory circuits and the immune effector mechanisms involved in this fascinating, still mysterious model of immunological therapy, our data suggest a novel high efficacy therapeutical tool against allergic asthma, but which might be transposed to other immune pathologies in which a cytokine imbalance is present.

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Table 1:

	Number of eosinophils/BALF (x 10^4)	Number of neutrophils/BALF (x 10^4)	Number of mononuclear cells/BALF (x 10^4)
Non-asthmatic control mice	0	0	2,328 ± 0,431
Sham-treated mice	20,188 ± 0,613	15,128 ± 0,598	7,234 ± 0,537
500 ng/dose IL-12-treated mice	0	0	1,452 ± 0,219
100 ng/dose IL-12-treated mice	0	0	2,473 ± 0,518
1 ng/dose non-activated IL-12-treated mice	0	2,377 ± 0,263	2,377 ± 0,376
10 pg/dose non-activated IL-12-treated mice	18,563 ± 0,743	11,648 ± 0,470	7,835 ± 0,681
100 fg/dose non-activated IL-12-treated mice	19,402 ± 0,438	14,931 ± 0,738	6,973 ± 0,872
1 fg/dose non-activated IL-12-treated mice	21,859 ± 0,370	13,422 ± 0,492	7,447 ± 0,333
10^{-2} fg/dose non-activated IL-12-treated mice	18,436 ± 0,817	15,872 ± 0,331	7,903 ± 0,518
10^{-4} fg/dose non-activated IL-12-treated mice	20,311 ± 0,579	14,652 ± 0,473	8,104 ± 0,231
1 ng/dose activated IL-12-treated mice	0	0	0,984 ± 0,481
10 pg/dose activated IL-12-treated mice	0	0	1,817 ± 0,532
100 fg/dose activated IL-12-treated mice	17,845 ± 0,663	7,943 ± 0,982	6,549 ± 0,496
1 fg/dose activated IL-12-treated mice	19,057 ± 0,810	13,744 ± 0,663	7,560 ± 0,631
10^{-2} fg/dose activated IL-12-treated mice	19,337 ± 0,447	15,389 ± 0,318	7,348 ± 0,537
10^{-4} fg/dose activated IL-12-treated mice	21,089 ± 0,327	16,270 ± 0,512	8,437 ± 0,491

Table 1. Evaluation of eosinophils, neutrophils and mononuclear cells infiltrate in BALFs from IL-12 treated mice. Data are presented as mean number of cells/BALF ± S.D..

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Table 2:

	Number of eosinophils/BALF (x 10^4)		Number of neutrophils/BALF (x 10^4)		Number of mononuclear cells/BALF (x 10^4)	
	Non-activated solution	**Activated solution**	**Non-activated solution**	**Activated solution**	**Non-activated solution**	**Activated solution**
Non-asthmatic control mice	0	-	0	-	2,328 ± 0,431	-
Sham-treated mice	20,188 ± 0,613	-	15,128 ± 0,598	-	7,234 ± 0,537	-
1 ng/dose	1,134 ± 0,267	0	2,760 ± 0,822	0	4,377 ± 0,518	1,453 ± 0,437
10 pg/dose	12,114 ± 0,481	0	5,643 ± 0,664	0	5,487 ± 0,371	2,365 ± 0,643
100 fg/dose	18,446 ± 0,508	0	13,218 ± 0,489	1,276 ± 0,318	7,890 ± 0,527	2,551 ± 0,318
1 fg/dose	18,567 ± 0,685	0	15,621 ± 0,876	1,314 ± 0,261	7,321 ± 0,712	1,984 ± 0,660
10^{-2} fg/dose	15,444 ± 0,622	16,567 ± 0,685	15,607 ± 0,654	9,788 ± 0,435	6,804 ± 0,438	5,431 ± 0,451
10^{-4} fg/dose	17,885 ± 0,912	18,736 ± 0,583	14,220 ± 0,827	14,672 ± 0,562	8,327 ± 0,622	14,886 ± 0,460

Table 2. Evaluation of eosinophilic infiltrate in BALFs from IL-12 plus IFN-γ treated mice. Data are presented as mean number of eosinophils/BALF ± S.D..

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Figure legends

Figure 1. Outline of the animal experimental protocol. The scheme shows the experimental protocol for induction of asthma model and oral cytokine treatment in mice.

Figure 2. Effects of daily treatment with 500 ng/dose recombinant IL-12 on murine asthma. The levels of different cytokines in serum samples and in BALFs was monitored throughout and at the end of treatment period, for evaluation of asthmatic state. In particular, levels of IL-4 (A), IL-5 (B), IL-12 (C) and IFN-γ (D) in mice sera were evaluated in treatment days 4^{th}, 7^{th}, 12^{th}, 16^{th} and 20^{th}, while BALFs were collected on day 20^{th} (E-H). In addition, mice maintained alive for an additional week after treatment end were evaluated for their serum levels of IL-12 (I) and IFN-γ (J). Data regarding non-asthmatic control mice, sham-treated mice and IL-12-treated mice are shown. All cytokine concentrations are expressed in pg/ml. Data are the means (±SD) of three independent experiments performed in triplicate. *, $p<0.01$ *vs* Sham-treated mice; **, $p<0.001$ vs Sham-treated mice (A-H) or vs control mice (I, J).

Figure 3. Effects of daily treatment with 100 ng/dose recombinant IL-12 on murine asthma. Graphs show the levels of IL-4 (A), IL-5 (B), IL-12 (C) and IFN-γ (D) in mice sera on day 7^{th} of treatment, and in BALFs on day 20^{th} (E-H). In addition, mice maintained alive for an additional week after treatment end were evaluated for their serum levels of IL-12 (I) and IFN-γ (J). Data regarding non-asthmatic control mice, sham-treated mice and IL-12-treated mice are shown. All cytokine concentrations are expressed in pg/ml. Data are the means (±SD) of three independent experiments performed in triplicate. *, $p<0.01$ vs Sham-treated mice (A-H) or vs control mice (I, J); **, $p<0.001$ vs Sham-treated mice.

Figure 4. Effects of daily treatment with non-activated low doses of recombinant IL-12 on murine asthma. Graphs show the levels of IL-4 (A), IL-5 (B), IL-12 (C) and IFN-γ (D) in mice sera on day 7^{th} of treatment, and in BALFs on day 20^{th} (E-H), for the different treatment groups. IL-12 concentrations in non-activated solutions were the following: 1 ng/dose (A), 10 pg/dose (B), 100 fg/dose (C), 1 fg/dose (D), 10^{-2} fg/dose (E) and 10^{-4} fg/dose (F). All cytokine concentrations are expressed in pg/ml. Data are the means (±SD) of three independent experiments performed in triplicate. *, $p<0.01$ vs Sham-treated mice. **, $p<0.001$ vs Sham-treated mice.

Figure 5. Effects of daily treatment with activated low doses of recombinant IL-12 on murine

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asthma. Graphs show the levels of IL-4 (A), IL-5 (B), IL-12 (C) and IFN-γ (D) in mice sera on day 7^{th} of treatment, and in BALFs on day 20^{th} (E-H), for the different treatment groups. IL-12 concentrations in activated solutions were the following: 1 ng/dose (A), 10 pg/dose (B), 100 fg/dose (C), 1 fg/dose (D), 10^{-2} fg/dose (E) and 10^{-4} fg/dose (F). All cytokine concentrations are expressed in pg/ml. Data are the means (±SD) of three independent experiments performed in triplicate. *, $p<0.01$ vs Sham-treated mice. **, $p<0.001$ vs Sham-treated mice.

Figure 6. Cytokine levels in mice sera treated with non-activated or activated low doses of recombinant IL-12 after end of treatment. Mice treated with 1 ng/dose non-activated (A) or 10 pg/dose activated (B) IL-12 solutions were maintained alive for an additional week after treatment end and were evaluated for their serum levels of IL-12 (A, C) and IFN-γ (B, D). Data regarding non-asthmatic control mice and IL-12-treated mice are shown. All cytokine concentrations are expressed in pg/ml. Data are the means (±SD) of three independent experiments performed in triplicate.

Figure 7. Effects of daily treatment with non-activated low doses of associated recombinant IL-12 and IFN-γ on murine asthma. Graphs show the levels of IL-4 (A), IL-5 (B), IL-12 (C) and IFN-γ (D) in mice sera on day 7^{th} of treatment, and in BALFs on day 20^{th} (E-H), for the different treatment groups who received co-delivery of IL-12 plus IFN-γ. IL-12 and IFN-γ concentrations in non-activated solutions were the following: 1 ng/dose (A), 10 pg/dose (B), 100 fg/dose (C), 1 fg/dose (D), 10^{-2} fg/dose (E) and 10^{-4} fg/dose (F). All cytokine concentrations are expressed in pg/ml. Data are the means (±SD) of three independent experiments performed in triplicate. *, $p<0.01$ vs Sham-treated mice. **, $p<0.001$ vs Sham-treated mice.

Figure 8. Effects of daily treatment with activated low doses of associated recombinant IL-12 and IFN-γ on murine asthma. Graphs show the levels of IL-4 (A), IL-5 (B), IL-12 (C) and IFN-γ (D) in mice sera on day 7^{th} of treatment, and in BALFs on day 20^{th} (E-H), for the different treatment groups who received co-delivery of IL-12 plus IFN-γ. IL-12 and IFN-γ concentrations in activated solutions were the following: 1 ng/dose (A), 10 pg/dose (B), 100 fg/dose (C), 1 fg/dose (D), 10^{-2} fg/dose (E) and 10^{-4} fg/dose (F). All cytokine concentrations are expressed in pg/ml. Data are the means (±SD) of three independent experiments performed in triplicate. *, $p<0.01$ vs Sham-treated mice. **, $p<0.001$ vs Sham-treated mice.

Figure 9. Histological analysis of lungs. Hematoxylin-eosin staining of lung parenchyma of

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control mice (A1), sham-treated mice (A2) and mice receiving 1 fg/die/mouse activated association of IL-12 and IFN-γ (A3) show presence of eosinophils (arrow heads) and enlarged macrophages (asterisk) only in the sham-treated group. Bars: 50 μm. Masson-trichrome staining shows an increase of fibrosis in sham-treated mice (B2), in comparison with control (B1) animals, while treatment with 1 fg/die/mouse activated association of IL-12 and IFN-γ significantly reduced the fibrotic accumulation (B3). Bars: 200 μm.

Figure 10. Effects of administration of 1 fg/die/mouse activated association of IL-12 and IFN-γ on different asthma-related parameters. Evaluation of serum antiovalbumin IgE, Balf IL-13 and serum IL-17 levels show the positive effects of 1 fg/die/mouse activated association of IL-12 and IFN-γ in our murine model of asthma. Data are the means (±SD) of three independent experiments performed in triplicate. *, $p<0.001$ vs Sham-treated mice.

Figure 11. Cytokine secretion by splenocytes *in vitro*. Graphs show the IL-12 (A) and IFN-γ (B) levels in spleen cell supernatants following *in vitro* treatment of splenocytes with low doses of activated IL-12 plus IFN-γ for 24 hours. The activated solutions of mixed IL-12 plus IFN-γ tested were the following: 1 pg/ml (C), 10 fg/ml (D), and 0.1 fg/ml (E). All cytokine concentrations are expressed in pg/ml. Data are the means (±SD) of two independent experiments performed in triplicate. *, $p<0.01$ vs control untreated cells. **, $p<0.001$ vs control untreated cells.

Figure 12. IL-12 secretion by CD11c+ dendritic cells *in vitro*. The graph shows the IL-12 levels in supernatants of CD11c+ isolated dendritic cells following *in vitro* treatment of dendritic cells with low doses of activated IL-12 plus IFN-γ for 24 hours. The activated solutions of mixed IL-12 plus IFN-γ tested were the following: 1 pg/ml (C), 10 fg/ml (D), and 0.1 fg/ml (E). IL-12 concentration is expressed in pg/ml. Data are the means (±SD) of two independent experiments performed in triplicate. *, $p<0.001$ vs control untreated cells.

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Table 1

	Number of eosinophils/BALF (x 10^4)	**Number of neutrophils/BALF (x 10^4)**	**Number of mononuclear cells/BALF (x 10^4)**
Non-asthmatic control mice	0	0	2,328 ± 0,431
Sham-treated mice	20,188 ± 0,613	15,128 ± 0,598	7,234 ± 0,537
500 ng/dose IL-12-treated mice	0	0	1,452 ± 0,219
100 ng/dose IL-12-treated mice	0	0	2,473 ± 0,518
1 ng/dose non-activated IL-12-treated mice	0	2,377 ± 0,263	2,377 ± 0,376
10 pg/dose non-activated IL-12-treated mice	18,563 ± 0,743	11,648 ± 0,470	7,835 ± 0,681
100 fg/dose non-activated IL-12-treated mice	19,402 ± 0,438	14,931 ± 0,738	6,973 ± 0,872
1 fg/dose non-activated IL-12-treated mice	21,859 ± 0,370	13,422 ± 0,492	7,447 ± 0,333
10^{-2} fg/dose non-activated IL-12-treated mice	18,436 ± 0,817	15,872 ± 0,331	7,903 ± 0,518
10^{-4} fg/dose non-activated IL-12-treated mice	20,311 ± 0,579	14,652 ± 0,473	8,104 ± 0,231
1 ng/dose activated IL-12-treated mice	0	0	0,984 ± 0,481
10 pg/dose activated IL-12-treated mice	0	0	1,817 ± 0,532
100 fg/dose activated IL-12-treated mice	17,845 ± 0,663	7,943 ± 0,982	6,549 ± 0,496
1 fg/dose activated IL-12-treated mice	19,057 ± 0,810	13,744 ± 0,663	7,560 ± 0,631
10^{-2} fg/dose activated IL-12-treated mice	19,337 ± 0,447	15,389 ± 0,318	7,348 ± 0,537
10^{-4} fg/dose activated IL-12-treated mice	21,089 ± 0,327	16,270 ± 0,512	8,437 ± 0,491

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Table 1. Evaluation of eosinophils, neutrophils and mononuclear cells infiltrate in BALFs from IL-12 treated mice. Data are presented as mean number of cells/BALF ± S.D..

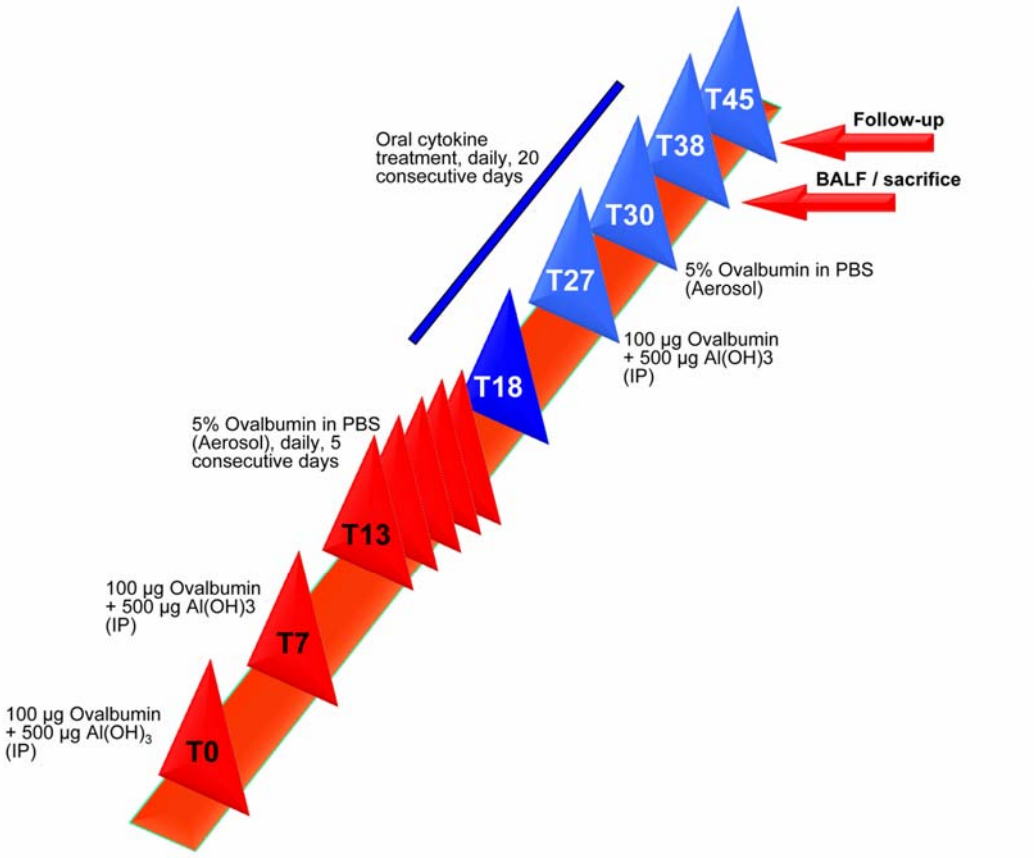
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Table 2

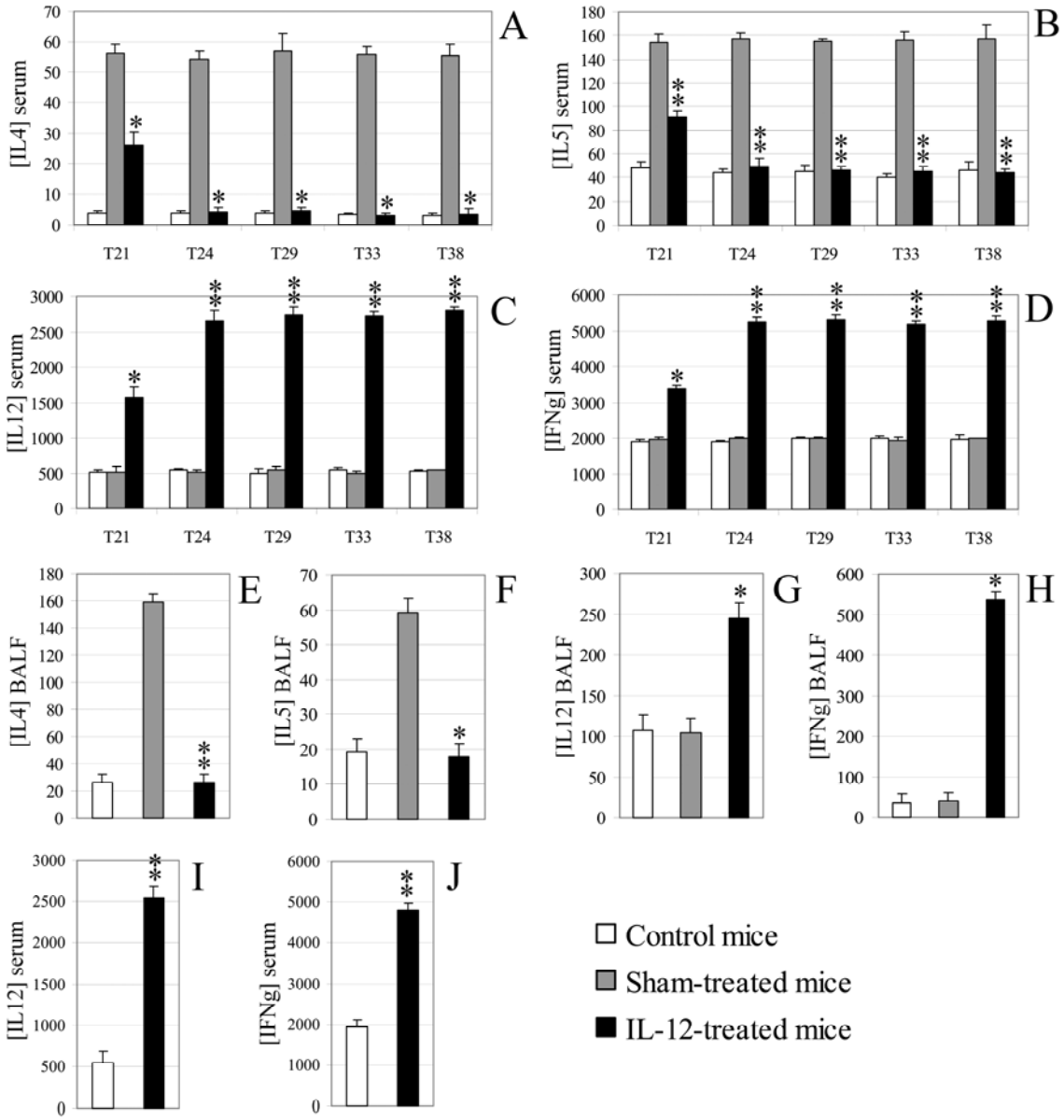
	Number of eosinophils/BALF (x 10^4)		Number of neutrophils/BALF (x 10^4)		Number of mononuclear cells/BALF (x 10^4)	
	Non-activated solution	**Activated solution**	**Non-activated solution**	**Activated solution**	**Non-activated solution**	**Activated solution**
Non-asthmatic control mice	0	-	0	-	2,328 ± 0,431	-
Sham-treated mice	20,188 ± 0,613	-	15,128 ± 0,598	-	7,234 ± 0,537	-
1 ng/dose	1,134 ± 0,267	0	2,760 ± 0,822	0	4,377 ± 0,518	1,453 ± 0,437
10 pg/dose	12,114 ± 0,481	0	5,643 ± 0,664	0	5,487 ± 0,371	2,365 ± 0,643
100 fg/dose	18,446 ± 0,508	0	13,218 ± 0,489	1,276 ± 0,318	7,890 ± 0,527	2,551 ± 0,318
1 fg/dose	18,567 ± 0,685	0	15,621 ± 0,876	1,314 ± 0,261	7,321 ± 0,712	1,984 ± 0,660
10^{-2} fg/dose	15,444 ± 0,622	16,567 ± 0,685	15,607 ± 0,654	9,788 ± 0,435	6,804 ± 0,438	5,431 ± 0,451
10^{-4} fg/dose	17,885 ± 0,912	18,736 ± 0,583	14,220 ± 0,827	14,672 ± 0,562	8,327 ± 0,622	14,886 ± 0,460

Table 2. Evaluation of eosinophilic infiltrate in BALFs from IL-12 plus IFN-γ treated mice. Data are presented as mean number of eosinophils/BALF ± S.D..

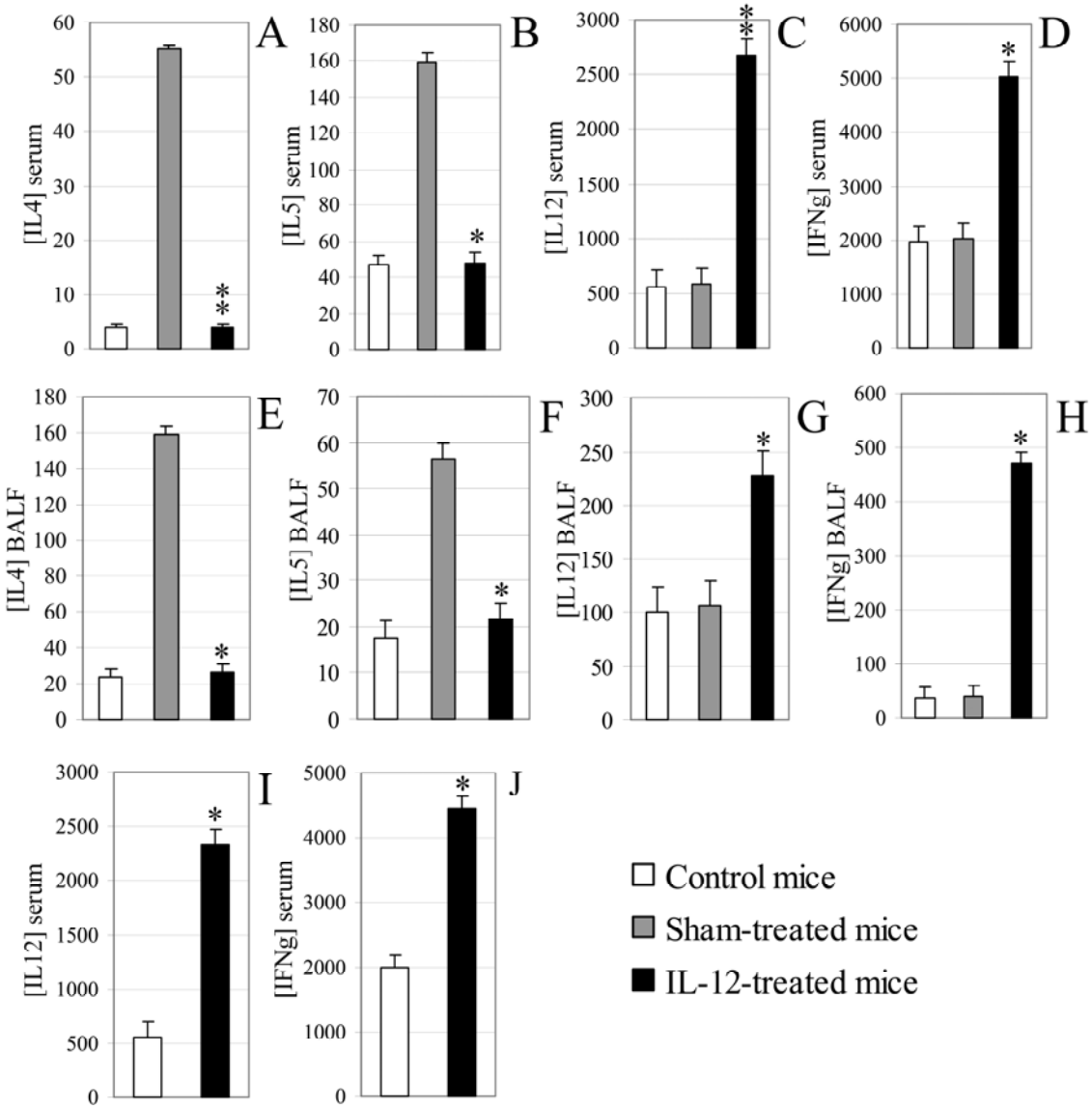
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T45
Follow-up
T38
BALF / sacrifice
T30
T27
5% Ovalbumin in PBS
(Aerosol)
Oral cytokine
treatment, daily, 20
consecutive days
100 µg Ovalbumin
+ 500 µg Al(OH)3
(IP)
T18
5% Ovalbumin in PBS
(Aerosol), daily, 5
consecutive days
T13
100 µg Ovalbumin
+ 500 µg Al(OH)3
(IP)
T7
100 µg Ovalbumin
+ 500 µg Al(OH)3
(IP)
T0

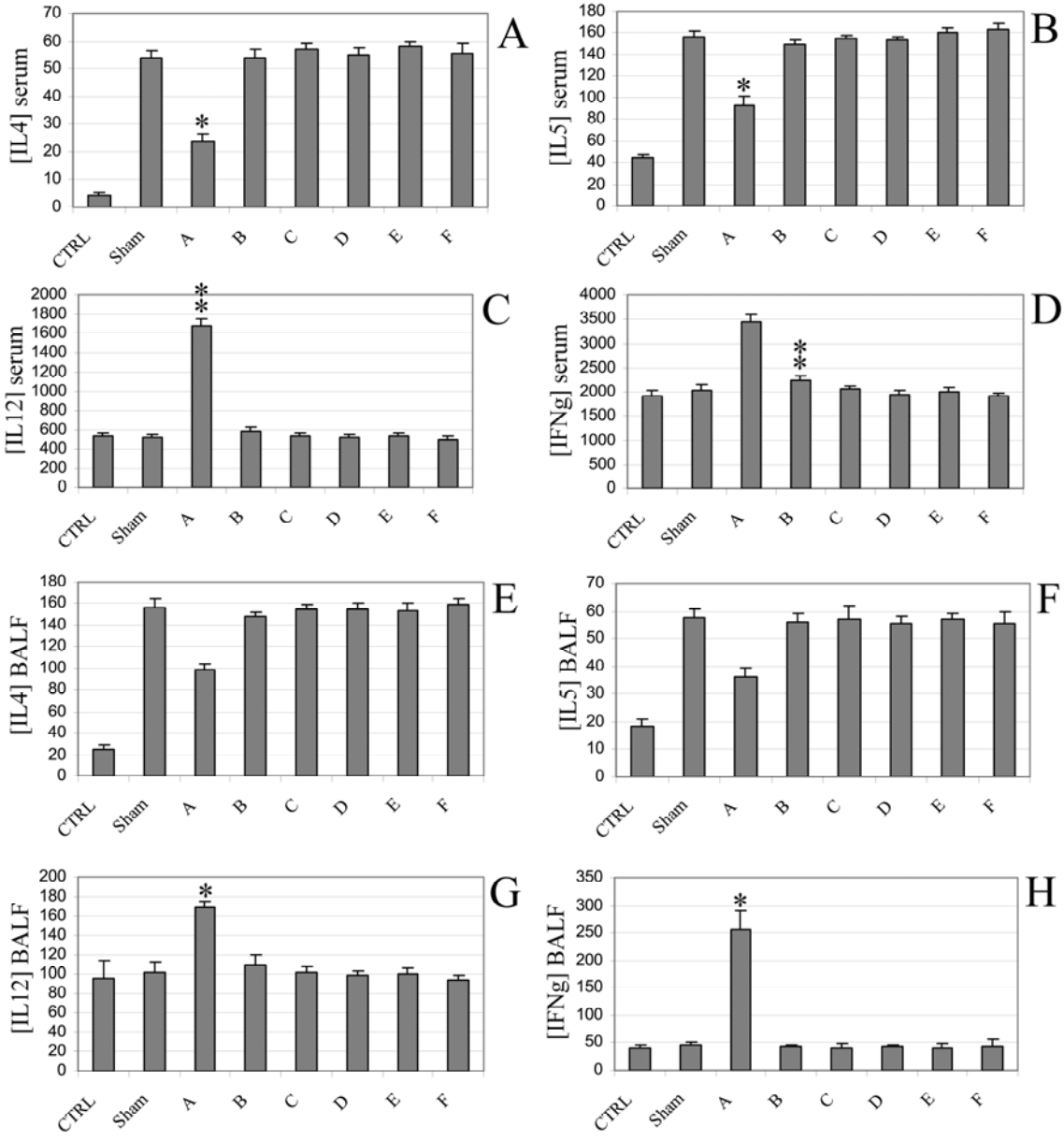
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B
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C
[IL12] serum
D
[IFNg] serum
T21
T24
T29
T33
T38
E
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F
[IL5] BALF
G
[IL12] BALF
H
[IFNg] BALF
I
[IL12] serum
J
[IFNg] serum
Control mice
Sham-treated mice
IL-12-treated mice

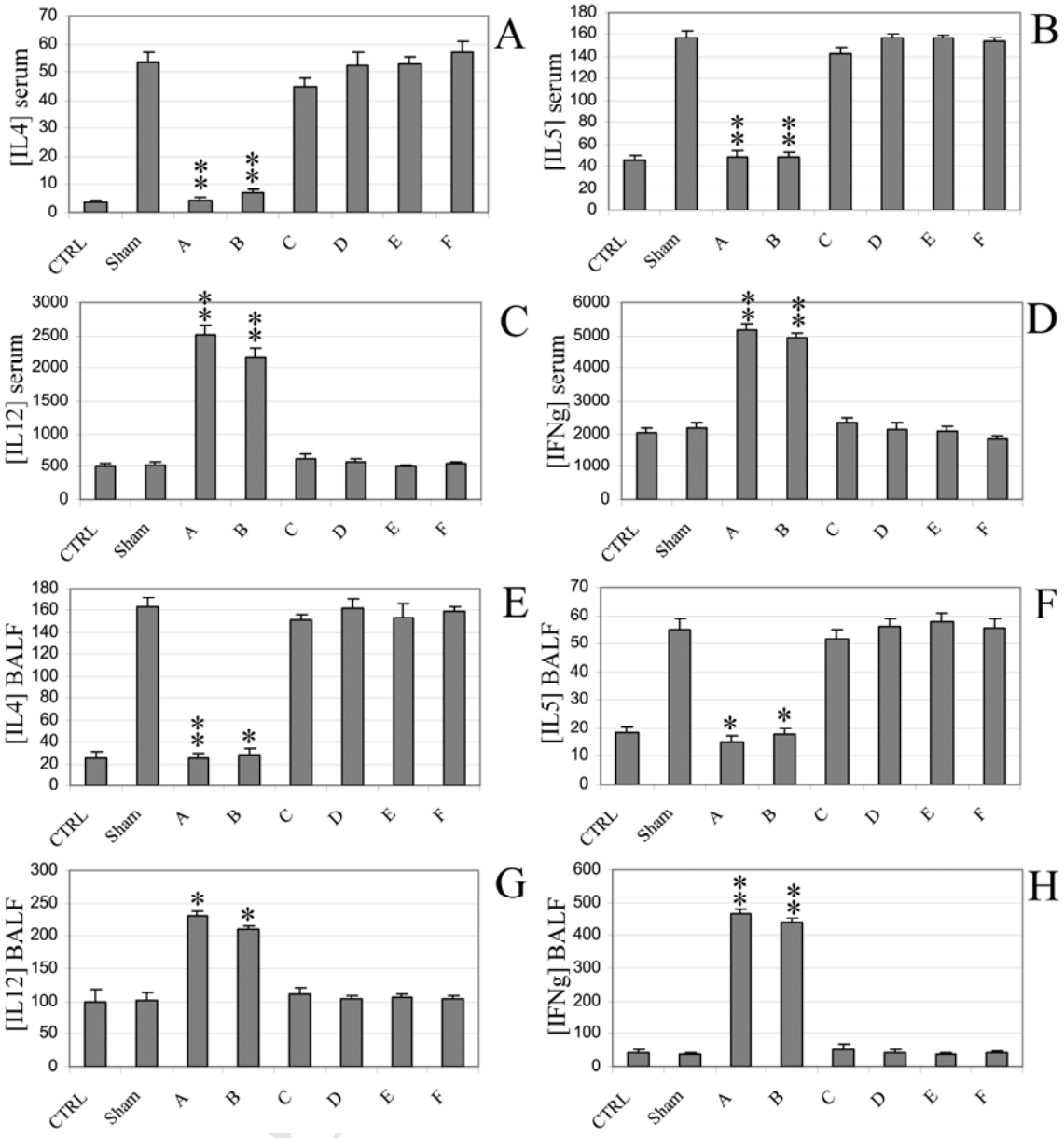
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G
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J
[IFNg] serum
Control mice
Sham-treated mice
IL-12-treated mice

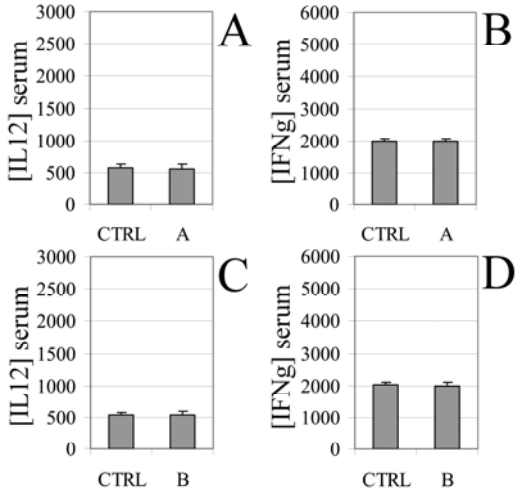
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C
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E
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Sham
A
B
C
D
E
F

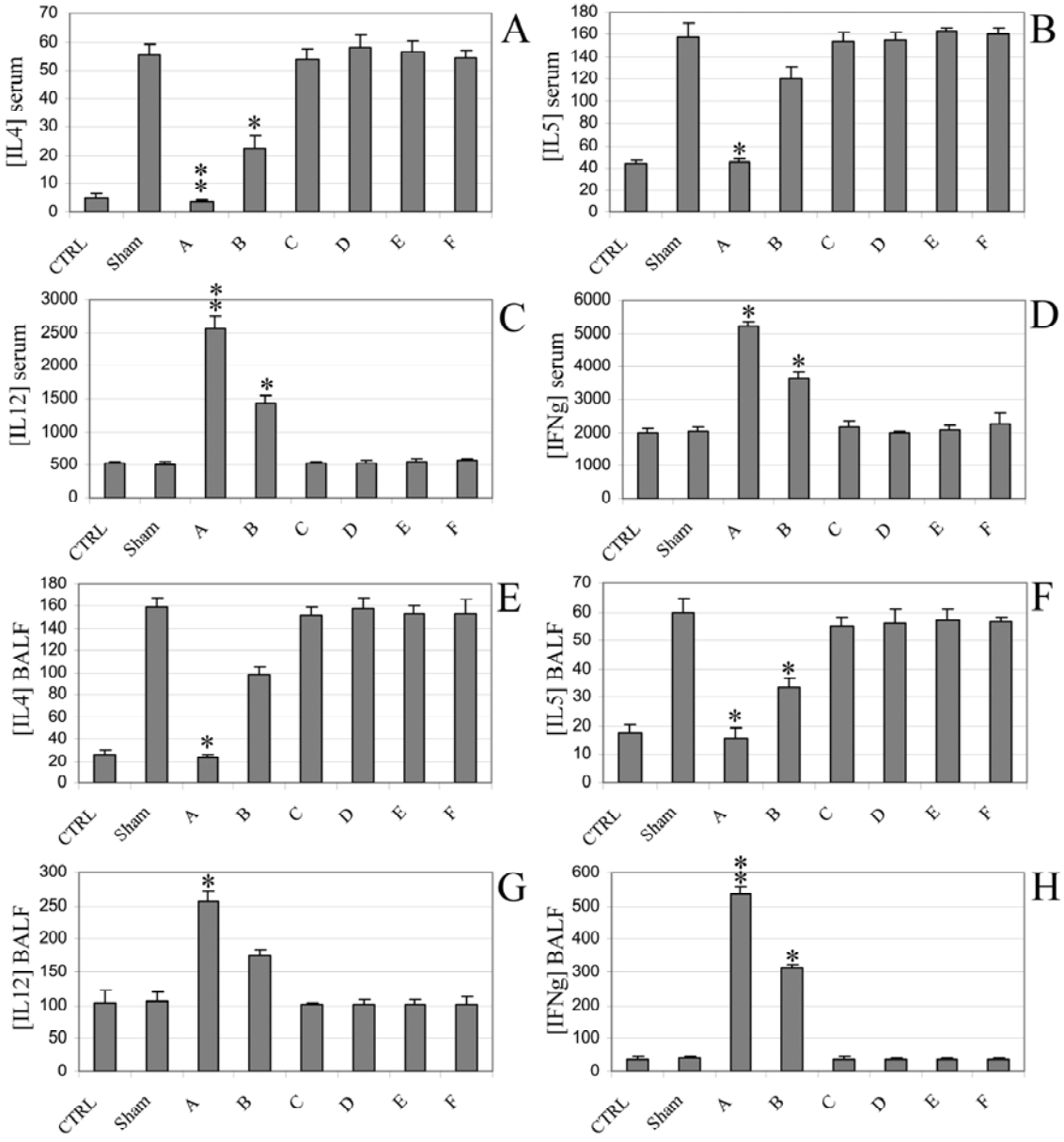
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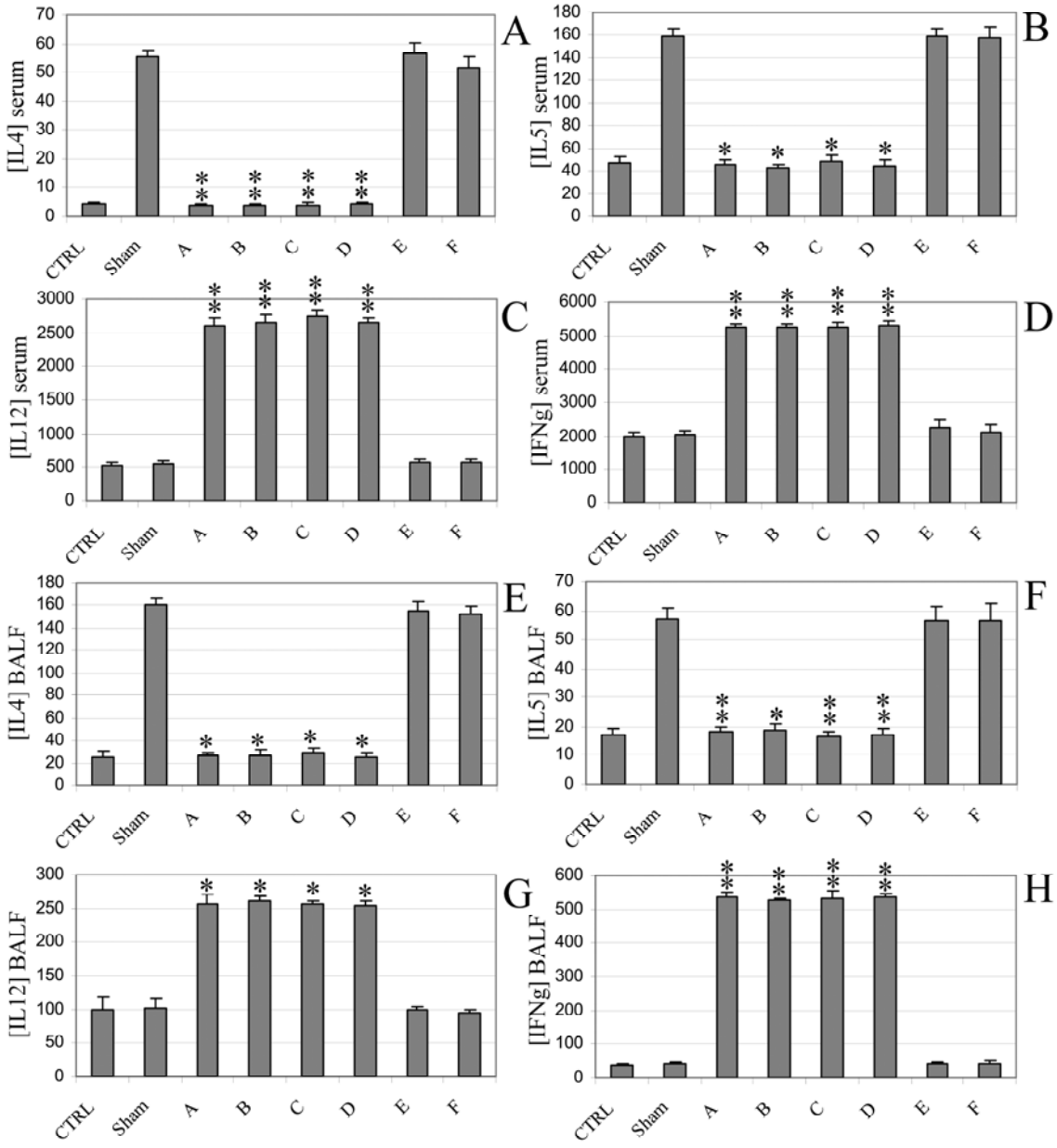

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H
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Sham
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C
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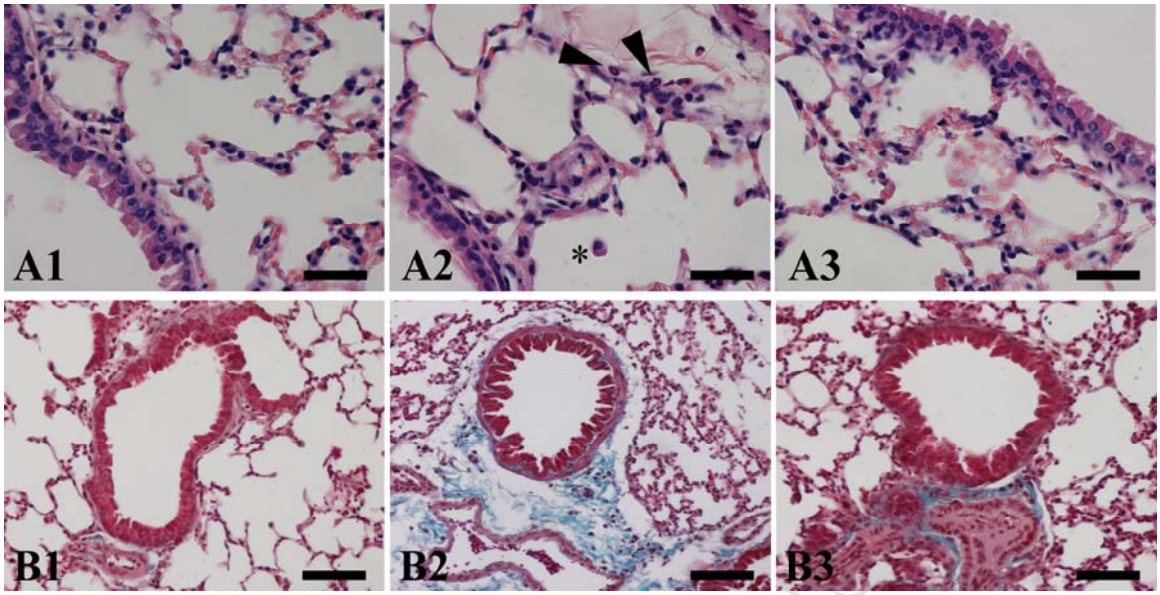


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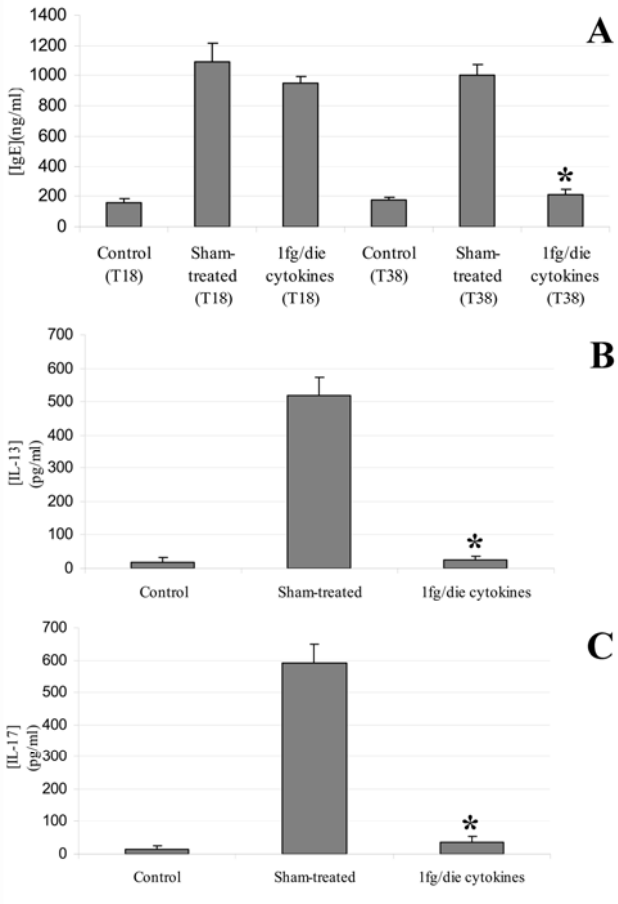

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50
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10
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Sham
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B
C
D
E
F
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140
120
100
80
60
40
20
0
C
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2500
2000
1500
1000
500
0
D
[IFNg] serum
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5000
4000
3000
2000
1000
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[IL4] BALF
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G
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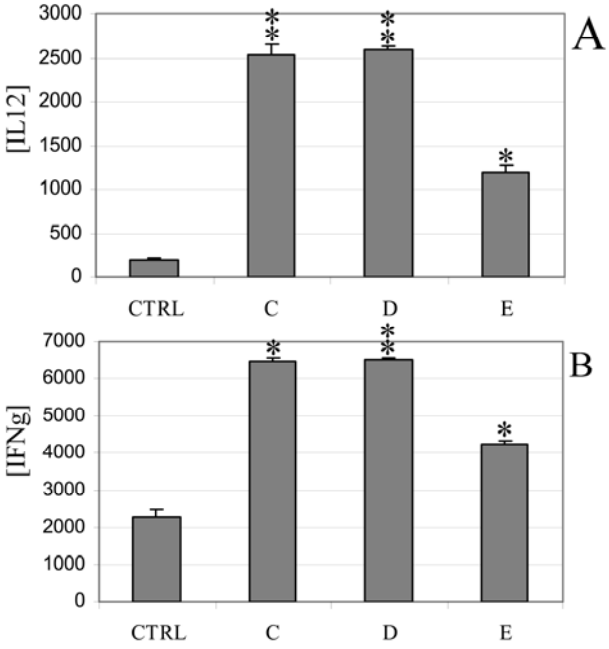
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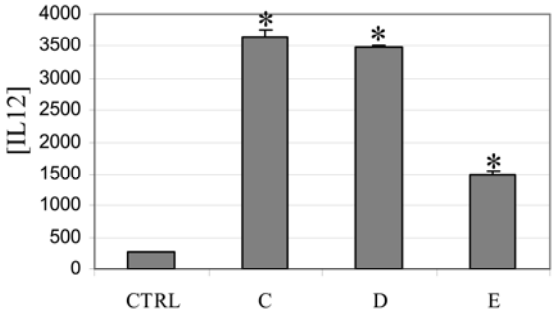
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