

Eosinophil-derived IFN- γ induces airway hyperresponsiveness and lung inflammation in the absence of lymphocytes

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Background: Eosinophils are key players in T_H2-driven pathologies, such as allergic lung inflammation. After IL-5- and eotaxin-mediated tissue recruitment, they release several cytotoxic and inflammatory mediators. However, their exact contribution to asthma remains controversial. Indeed, in human subjects anti-IL-5 treatment inhibits eosinophilia but not antigen-induced airway hyperresponsiveness (AHR). Likewise, lung fibrosis is abrogated in 2 strains of eosinophil-deficient mice, whereas AHR is inhibited in only one of them. Finally, eosinophils have been shown to attract T_H2 lymphocytes at the inflammatory site.

Objective: The ability of eosinophils to promote AHR and lung inflammation independently of lymphocytes was investigated.

Methods: Adoptive transfers of resting or activated eosinophils from IL-5 transgenic mice were performed into naive BALB/c mice, mice with severe combined immunodeficiency, and IFN- γ -deficient BALB/c recipients.

Results: Adoptively transferred eosinophils induced lung inflammation, fibrosis, collagen deposition, and AHR not only in BALB/c mice but also in recipient mice with severe combined immunodeficiency. Surprisingly, IFN- γ expression was increased in lungs from eosinophil-transferred animals. Furthermore, IFN- γ neutralization in recipients partially inhibited eosinophil-induced AHR. Moreover, IFN- γ -deficient eosinophils or eosinophils treated with a blocking anti-IFN- γ receptor antibody failed to induce AHR in IFN- γ -deficient recipients. Finally, *in vitro* and at low concentrations, IFN- γ increased eosinophil peroxidase release, potentiated chemotaxis,

and prolonged survival, suggesting the existence of an autocrine mechanism.

Conclusions: These results support the important and previously unsuspected contribution of eosinophils to lung inflammation independently of lymphocytes through production of IFN- γ , the prototypical T_H1 cytokine. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Adoptive transfer, airway hyperresponsiveness, asthma, eosinophil, IFN- γ , lung inflammation, severe combined immunodeficiency

Eosinophils are usually associated with T_H2-associated pathologies, such as helminth infections or allergies, and IgE-dependent activation. Indeed, IL-5 and eotaxins are the main eosinophil differentiation and chemotactic factors. Through the release of cytotoxic mediators, in particular cationic proteins (eosinophil peroxidase [EPO], major basic protein, eosinophil cationic protein, and eosinophil derived neurotoxin), and generation of reactive oxygen species,¹ eosinophils not only contribute to cytotoxicity toward parasites but also to the tissue damage observed in allergic inflammation. Eosinophil-derived cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-16, IL-18 and TGF- β), chemokines (RANTES and eotaxin 1), and lipid mediators (platelet-activating factor and leukotriene C₄) can exacerbate allergic inflammation.¹ Importantly, eosinophils are critical for asthma-associated airway fibrosis and remodeling, as demonstrated with 2 strains of eosinophil-deficient animals.^{2,3} Furthermore, eosinophils also act as regulators of the T_H1/T_H2 balance through IFN- γ and IL-10 production⁴ or as antigen-presenting cells.⁵

However, the exact contribution of eosinophils to lung inflammation and function remains controversial. Indeed, in asthmatic patients treatment with anti-IL-5 suppresses eosinophilia but not allergen-induced airway hyperresponsiveness (AHR),⁶ whereas AHR is restored upon eosinophil transfer into IL-5-deficient recipient mice.⁷ Likewise, AHR is inhibited in only one of the 2 strains of eosinophil-deficient mice.^{2,3} Along the same lines, eosinophils appear essential for lung recruitment of T_H2 lymphocytes through production of T_H2 chemokines, such as macrophage-derived chemokine/CCL2 and thymus and activation-regulated chemokine/CCL17,⁸ but this contribution is dependent on the genetic background (found in C57BL/6 mice but not in BALB/c mice) and might also involve other molecules, such as monocyte chemotactic protein 3 and eotaxin 1 and 2.⁹ In an even more contrasting way, eosinophilia (in IL-5 transgenic

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Abbreviations used

AHR:	Airway hyperresponsiveness
BALF:	Bronchoalveolar lavage fluid
Eos 1×:	Single transfer of eosinophils
Eos 4×:	Four eosinophil transfers
EPO:	Eosinophil peroxidase
IFN-γR1:	IFN-γ receptor 1
IFN-γR2:	IFN-γ receptor 2
OVA:	Ovalbumin
SCID:	Severe combined immunodeficiency
Tg:	Transgenic

[Tg] mice) prevents AHR development¹⁰ and leads to attenuation of the type 2 immune response.¹¹

Furthermore, in human subjects, even though anti-IL-5 antibodies efficiently decrease eosinophilia in asthmatic patients⁶ and in a limited number of hypereosinophilic syndromes,¹² treatments have usually failed to cure asthma.^{13,14}

Because of these conflicting results and reasoning that an early eosinophil contribution to lung inflammation might have been blurred by the concomitant effect of other cell types, we have thus attempted to delineate the specific eosinophil contribution to lung inflammation using adoptive transfer into naive recipients.

METHODS**Animals**

BALB/c mice were from Janvier (Le Genest-Saint-Isle, France), and CB-17 severe combined immunodeficiency (SCID) mice were from the breeding colony at the Institut Pasteur de Lille. IFN-γ^{-/-} (BALB/c) mice¹⁵ and IL-5 Tg (BALB/c) mice¹⁶ were bred within the specific pathogen-free animal facility from the Institut Pasteur de Lille. IFN-γ^{-/-} X IL-5 Tg mice were obtained after 2 generations. All the experiments were performed after approval by the ethics committee for animal experimentation from the Nord-Pas de Calais Region.

Eosinophil purification and transfer

Eosinophils were from IFN-γ^{+/+} X IL-5 Tg or IFN-γ^{-/-} X IL-5 Tg (8–12 weeks old) mice sensitized by means of intraperitoneal injection of 50 μg of ovalbumin (OVA) in 100 μL of alum solution at days 0, 7, and 14 (control animals received alum only) and killed at day 15 (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Eosinophils were purified by means of negative selection with biotin-conjugated anti-CD19, anti-CD90.2, and anti-CD8α antibodies (BD PharMingen, San Jose, Calif) and anti-biotin-conjugated magnetic beads, as previously described.¹⁷ Purity was greater than 90%. One to 4 × 10⁷ purified eosinophils or vehicle alone were transferred to naive BALB/c, SCID, or IFN-γ^{-/-} recipient mice (8–10 weeks old) by means of intratracheal administration (see Fig E1). In some experiments purified eosinophils were labeled with carboxyfluorescein succinimidyl ester before transfer. Lung function was evaluated 1, 2, 3, 4, and 7 days after eosinophil transfer. In some experiments recipient mice received anti-mouse IFN-γ antibody (clone XMG1.2, 0.5 mg per mouse) intraperitoneally 24 hours before transfer. Recipient mice were killed, and bronchoalveolar lavage fluid (BALF) was collected. Right and left lungs were collected for RT-PCR and collagen assay, respectively. After repeated eosinophil transfers, serum was collected, and IgE concentrations were measured by means of ELISA.¹⁸

For RT-PCR analysis, eosinophils from IL-5 Tg mouse spleens were purified by means of cell sorting on a FACS Aria (Becton Dickinson, Mountain View, Calif) on the basis of their forward and side scatter parameters after labeling with anti-Gr-1-allophycocyanin and anti-CD19-phycoerythrin, anti-CD90.2-fluorescein isothiocyanate. Purity was 99.5%.

Invasive plethysmography

Measurement of dynamic lung resistance and compliance were performed as previously described¹⁹ with Flexivent (SCIREQ, Montreal, Quebec, Canada) after eosinophil transfer. Briefly, recipient mice were anesthetized with 0.5 mg/kg medetomidine (Domitor; Pfizer, New York, NY) and 50 mg/kg ketamine (Imalgene 1000; Merial) and surgically intubated with a 20-gauge catheter (Introcan; B. Braun, Melsungen, Germany). Animals then received 0.1 mg/kg pancuronium bromide (Pavulon; Organon International, Oss, The Netherlands) before mechanical ventilation. Increasing methacholine concentrations (0, 3, 6, 12, and 24 mg/mL in PBS) were administered through an electronic nebulizer (Aeroneb; Aerogen, Galway, Ireland).

Eosinophil function *in vitro*

For EPO release, eosinophils (3 × 10⁵/100 μL of RPMI per well) were re-suspended in RPMI 1640, and incubated with 1 to 100 pg/mL IFN-γ or PBS for 2 hours at 37°C. Eosinophil supernatants or BALF were transferred into a microtiter plate, and luminol (50 μg/mL), D-luciferin (4 × 10⁻⁵ mol/L; Roche Diagnostics, Mannheim, Germany), and H₂O₂ were injected. Chemiluminescence was immediately measured with a luminometer (Victor; PerkinElmer, Waltham, Mass).

For the survival assay, eosinophils (1.10⁶/250 μL of RPMI with 10% mouse serum per well) were incubated with 1 to 1000 pg/mL IFN-γ or PBS overnight at 37°C and stained with annexin V-fluorescein isothiocyanate and 7-amino-actinomycin D (Sigma, St Louis, Mo) for 15 minutes at room temperature. Samples were analyzed by means of flow cytometry. Double-negative annexin V and 7-amino-actinomycin D were considered as surviving cells.

Chemotaxis assay was performed in a Boyden Chamber by using eotaxin (R&D Systems, Minneapolis, Minn) as a chemoattractant. IFN-γ (1–1000 pg/mL) was added to IFN-γ^{+/+} or IFN-γ^{-/-} eosinophils immediately before the deposition of eosinophils in the upper chamber, and the priming effect on eotaxin-induced eosinophil chemotaxis was examined after 90 minutes' incubation at 37°C.

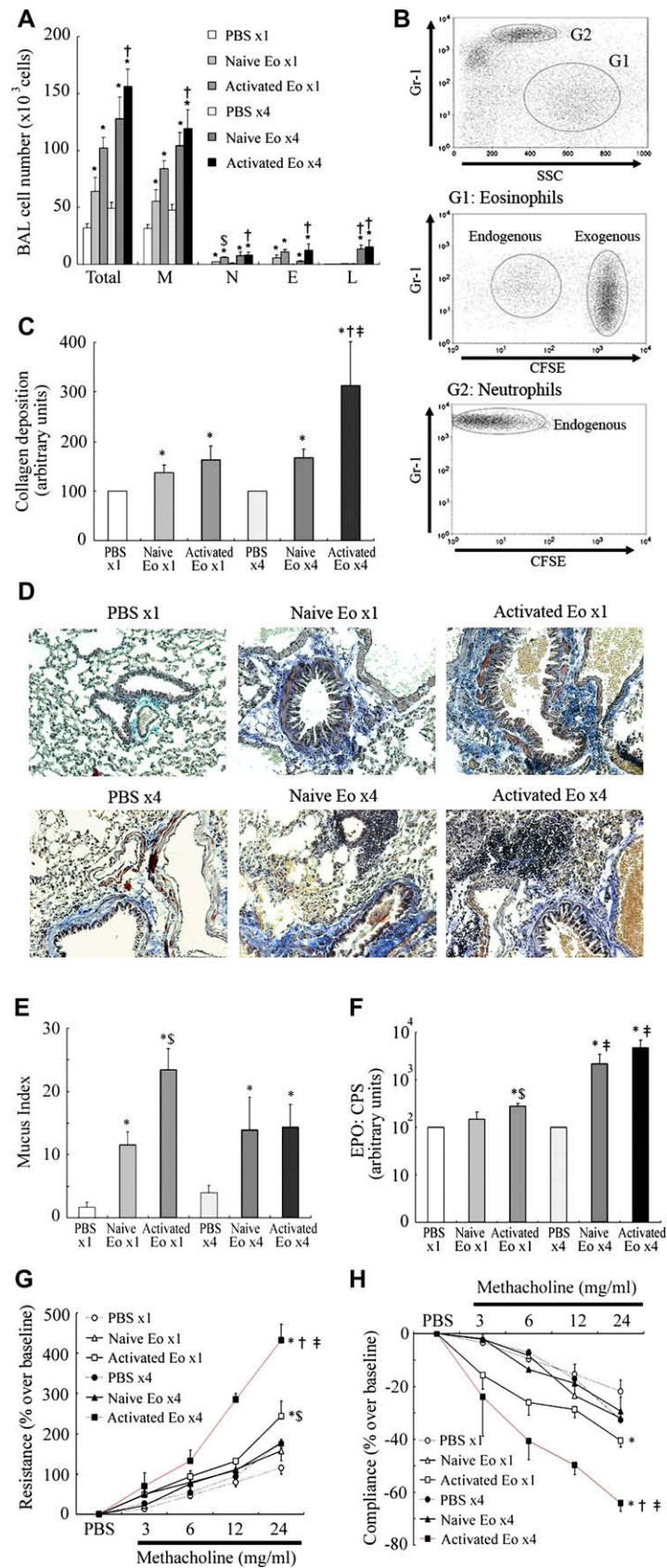
Statistical analyses

Data are presented as means ± SEMs. Statistical significance was determined by using the Mann-Whitney test, except for invasive plethysmographic data, for which repeated-measures ANOVA was used.

A supplementary Methods section can be found in this article's Online Repository.

RESULTS**Eosinophils induce lung inflammation and AHR in BALB/c mice**

Taking into account a potential contribution of eosinophils to early phases of the airway inflammatory process, we first adoptively transferred highly purified splenic eosinophils (2 × 10⁷ cells) from naive or antigen-sensitized IL-5 Tg animals to naive BALB/c recipients that were killed 72 hours later (see Fig E1, A). After a single transfer of naive or activated eosinophils (Eos 1×), a significant recruitment of inflammatory cells (eosinophils, neutrophils, and macrophages) was detected in BALF (Fig 1, A). Activated Eos 1× induced higher neutrophilia than naive Eos 1×, whereas lymphocyte recruitment was only marginal. Naive or activated Eos 1× did not induce a significant difference in BALF eosinophilia. Carboxyfluorescein succinimidyl ester labeling of eosinophils before the transfer evidenced that BALF contained both transferred and endogenously recruited eosinophils in addition to neutrophils and macrophages at the time of death (Fig 1, B). Fibrosis is also considered a hallmark of chronic airway remodeling. Naive or activated Eos 1× significantly increased eosinophils' collagen deposition,



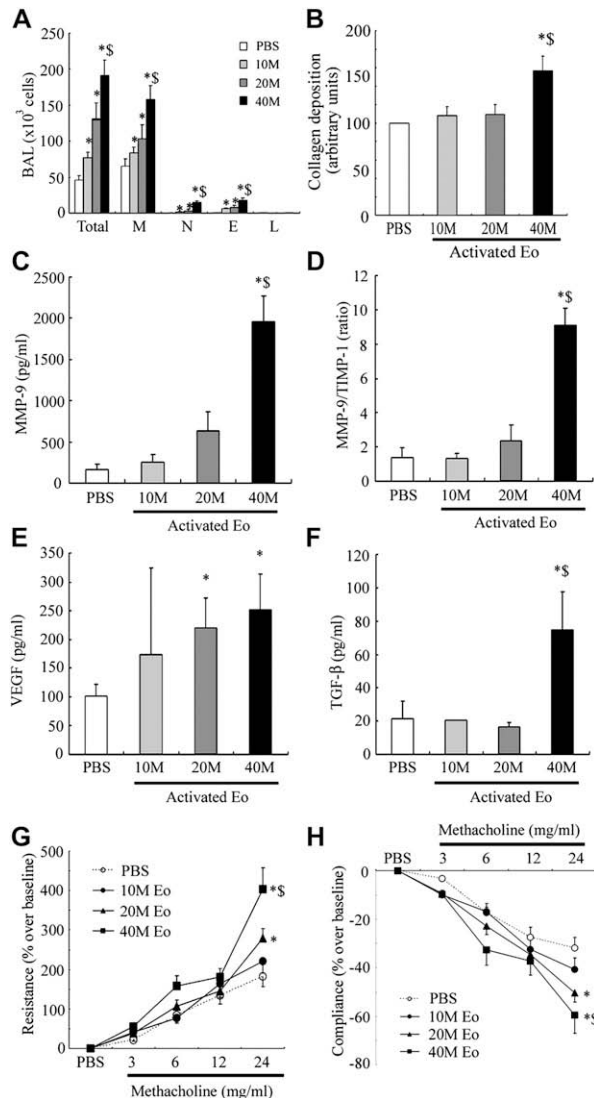


FIG 2. Activated eosinophils induce number-dependent lung inflammation in SCID recipient mice. PBS, and 10^7 , 2×10^7 , or 4×10^7 activated eosinophils (Eo) were transferred to SCID mice, and the animals were killed 72 hours later. **A**, BALF cellularity ($n = 5-9$ per group). **B**, Collagen deposition ($n = 7-14$ per group). **C-F**, Lung production of matrix metalloproteinase 9 (MMP-9; Fig 2, C), matrix metalloproteinase 9/tissue inhibitor of metalloproteinase 1 ratio (MMP-9/TIMP-1; Fig 2, D), vascular endothelial growth factor (VEGF; Fig 2, E), and TGF- β (Fig 2, F) in BALF is quantified ($n = 3-7$). **G** and **H**, Airway resistance (Fig 2, G) and compliance (Fig 2, H; $n = 5-10$ per group). *Statistically different from PBS-treated recipient mice ($P < .05$). \$Statistically different from 10^7 activated eosinophils ($P < .05$).

reflecting fibrosis (Fig 1, C). In lung tissue naive Eos $1 \times$ induced a minor peribronchial recruitment of inflammatory cells, whereas activated Eos $1 \times$ induced a more pronounced recruitment (Fig 1, D). Naive or activated Eos $1 \times$ also induced a significant mucus

accumulation, a characteristic feature of allergic asthma (see Fig E2, A, in this article's Online Repository at www.jacionline.org).²⁰ The mucus index was about twice higher with activated Eos $1 \times$ compared with naive Eos $1 \times$ (Fig 1, E). Furthermore, naive Eos $1 \times$ increased EPO activity in BALF. This activity was further enhanced with activated Eos $1 \times$ (Fig 1, F). Compared with results seen in vehicle-treated recipients (PBS), naive Eos $1 \times$ failed to induce changes in methacholine-induced AHR, as assessed by measurements of airway resistance and compliance. By contrast, activated Eos $1 \times$ significantly increased resistance and decreased compliance (Fig 1, G and H). To further confirm our previous observations, we investigated whether repeated transfers would increase eosinophil-induced airway inflammation. Indeed, upon 4 eosinophil transfers (Eos $4 \times$), a more pronounced peribronchial and perivascular inflammation was observed, in particular for activated Eos $4 \times$ (Fig 1, D). Lymphocytes were also significantly recruited in BALF in addition to neutrophils, eosinophils, and macrophages (Fig 1, A). Naive Eos $4 \times$ increased collagen deposition, mucus, and EPO by 167%, 349%, and 222%, respectively, but did not affect AHR compared with that seen in corresponding PBS-treated animals. Activated Eos $4 \times$ increased collagen deposition, mucus accumulation, and EPO activity in BALF by 314%, 359%, and 4815%, respectively, compared with that seen in PBS-treated animals (Fig 1, C, E, and F). Activated Eos $4 \times$ increased airway resistance and decreased compliance by 432% and 64%, respectively, compared with baseline values (Fig 1, G and H). Corresponding changes for activated Eos $1 \times$ were 244% and 40%, respectively.

Our results suggest that upon transfer to naive recipients, eosinophils alone, in particular after exogenous activation, are able to induce many of the morphologic and functional characteristics of allergic inflammation.

Eosinophils induce lung inflammation in the absence of lymphocytes

Because eosinophil-induced lymphocyte recruitment in bronchoalveolar lavage airway lumen was marginal (Fig 1, A) and IgE levels remained very low and comparable with values seen in vehicle-treated animals even with activated Eos $4 \times$ (data not shown), we examined whether lymphocytes were dispensable for eosinophil-induced lung inflammation. Development of AHR was monitored over a 1-week period after adoptive transfer to naive SCID mice of 2×10^7 activated eosinophils. Compared with PBS-treated animals, no increase in AHR was observed 24 or 48 hours after transfer. By contrast, 72 and 96 hours after transfer, AHR was significantly increased, whereas lung tissue inflammation was barely present (see Fig E2, B). Such an effect on AHR had disappeared by day 7 (see Fig E3, A, in this article's Online Repository at www.jacionline.org). Although eosinophil transfer had little effect on histologic changes and mucus accumulation in SCID mouse lungs (see Figs E2, B, and E4, D, in this article's Online Repository at www.jacionline.org), it led to eosinophil,

FIG 1. Transfer of activated eosinophils induces lung inflammation in BALB/c recipient mice. PBS or naive or activated eosinophils (Eo) were transferred once ($\times 1$) or 4 times ($\times 4$) to BALB/c mice, and animals were killed 72 hours later. **A**, BAL cellularity: macrophages (M), neutrophils (N), eosinophils (E), and lymphocytes (L; $n = 6-9$ per group). **B**, Identification of carboxyfluorescein succinimidyl ester (CFSE)-labeled injected eosinophils and endogenous eosinophils and neutrophils. SSC, side scatter. **C**, Collagen deposition ($n = 6-20$ per group). **D**, Lung inflammation after 1 or 4 eosinophil transfers (original magnification $\times 100$). **E**, Mucus index ($n = 3-5$ per group). **F**, EPO activity in BALF. **G** and **H**, Airway resistance (Fig 1, G) and compliance (Fig 1, H; $n = 3-5$ per group). Open and solid symbols, 1 and 4 transfers, respectively. Statistically different ($P < .05$) from *PBS-treated recipient mice, \$naive $1 \times$ eosinophils, † $1 \times$ activated eosinophils, and ‡ $4 \times$ naive eosinophils.

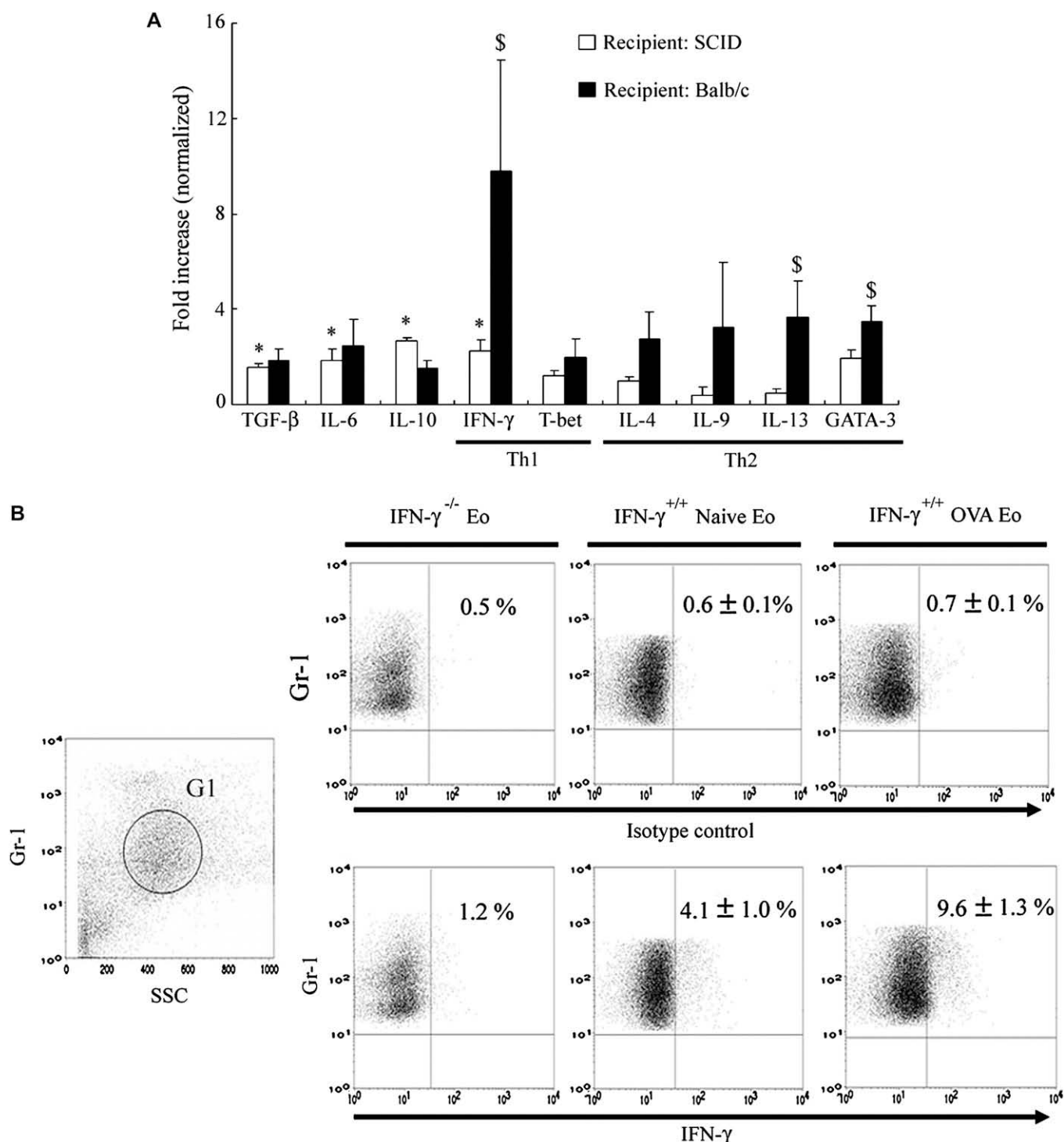


FIG 3. IFN- γ contributes to eosinophil-induced lung inflammation. **A** and **B**, Cytokine and transcription factor mRNA expression in lungs from BALB/c (**Fig 3, A**) and SCID (**Fig 3, B**) recipient mice measured 72 hours after transfer of 2×10^7 activated eosinophils (Eo). Results are expressed as the mean fold induction over that seen in PBS-treated recipient mice ($n = 4$). SSC, Side scatter. **C**, Intracellular IFN- γ content of eosinophils (Gr-1^{low}) evaluated by means of flow cytometry after a 6-hour brefeldin A treatment ($n = 3-5$). *Statistically different from PBS-treated recipient mice ($P < .05$).

neutrophil, and macrophage recruitment in airway lumen. Such recruitment was dependent on the number of transferred eosinophils (**Fig 2, A**). Indeed, transfer of 4×10^7 eosinophils not only further increased BALF inflammatory infiltrate but also

fibrosis (**Fig 2, A and B**). Nevertheless, in agreement with the major role played by lymphocytes in lung inflammation, collagen deposition, mucus accumulation and airway resistance induced in SCID mice were significantly lower and compliance was higher

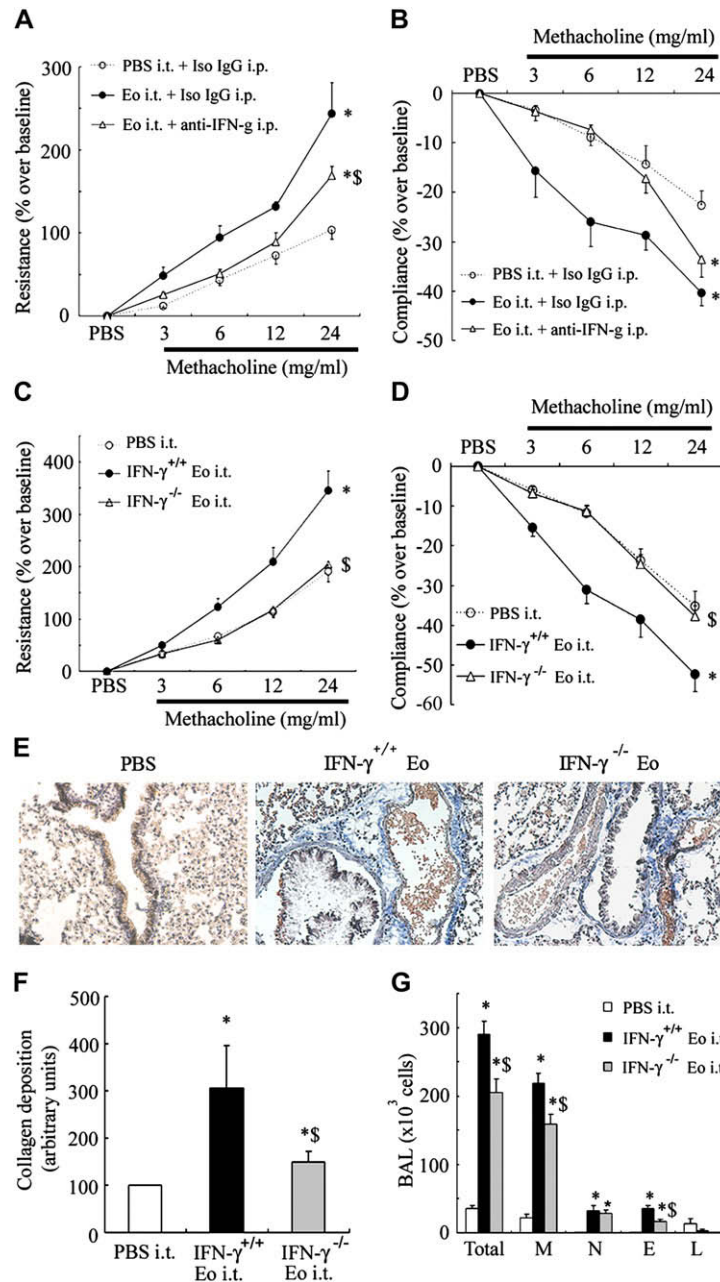
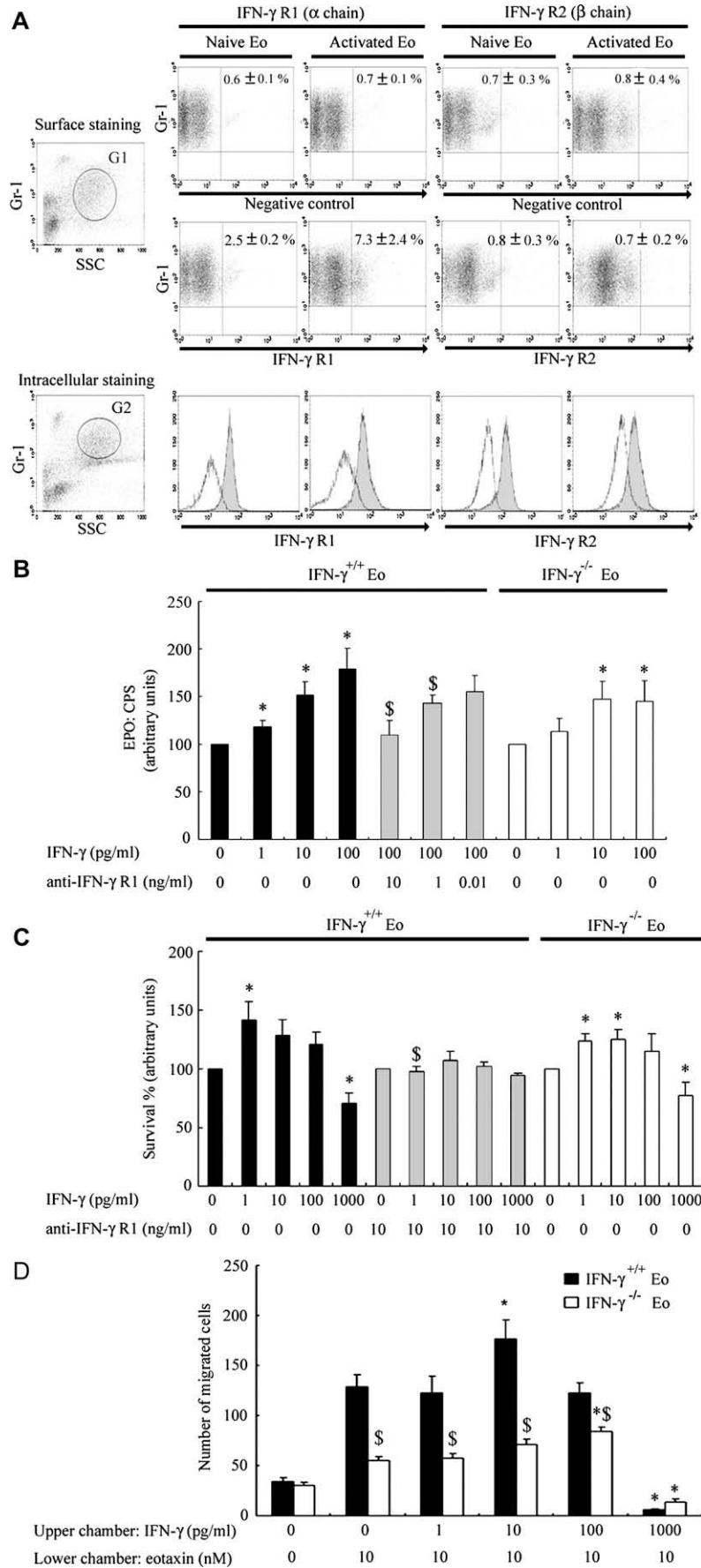


FIG 4. Eosinophil-derived IFN- γ is essential for eosinophil-induced lung inflammation. **A** and **B**, Airway resistance (Fig 4, A) and compliance (Fig 4, B; $n = 4-9$) in recipient BALB/c mice after PBS administration or transfer of activated eosinophils and injection with isotype (Iso) IgG, anti-mouse IFN- γ antibody, or both 24 hours before the transfer of activated eosinophils (Eo). *i.t.*, Intratracheal. **C-G**, AHR and inflammation 72 hours after transfer of IFN- $\gamma^{+/+}$ or IFN- $\gamma^{-/-}$ activated eosinophils to IFN- $\gamma^{-/-}$ recipient mice. Airway resistance (Fig 4, C) and compliance (Fig 4, D) after PBS, IFN- $\gamma^{+/+}$, and IFN- $\gamma^{-/-}$ activated eosinophil administration are shown ($n = 9-13$ per group). **E**, Lung inflammation in IFN- $\gamma^{-/-}$ recipient mice (original magnification $\times 100$). **F**, Collagen deposition ($n = 10$). **G**, BALF cellularity: macrophages (M), neutrophils (N), eosinophils (E), and lymphocytes (L; $n = 9-10$). Statistically different ($P < .05$) from *PBS-treated recipient mice and §iso-type IgG-treated or IFN- $\gamma^{+/+}$ transferred eosinophils ($P < .05$).

than that induced in BALB/c recipients upon transfer with the same number of activated eosinophils (see Fig E4).

Among other cell types, eosinophils are able to produce molecules involved in fibrosis and airway remodeling.²⁰ Transfer of activated eosinophils increased matrix metalloproteinase 9, the ratio of matrix metalloproteinase 9 and tissue inhibitor of

metalloproteinase 1, vascular endothelial growth factor, and TGF- β concentrations in BALF in a dose-dependent manner (Fig 2, C-F). Furthermore, compared with baseline values, airway resistance significantly increased (279% and 403%) and compliance decreased (50% and 60%) upon transfer of 2 and 4×10^7 activated eosinophils (Fig 2, G and H).



Thus our data further indicate that although lymphocytes contribute to lung inflammation, activated eosinophils induce inflammatory cell recruitment, fibrosis, and AHR in naive animals, probably in part through the direct and/or indirect release of profibrotic factors.

Eosinophil transfer leads to IFN- γ production

To get insight on the immune response induced in lung tissue on transfer of activated eosinophils, we analyzed, by means of real-time PCR, the expression of various factors involved in T_H2 , T_H1 , inflammatory, and regulatory responses. To our surprise, a strong induction of IFN- γ expression was detected in lungs from BALB/c mice 72 hours after a single transfer of activated eosinophils (Fig 3, A). Induction of factors associated with T_H2 , inflammatory, or regulatory responses, such as IL-13, IL-4, IL-9, IL-6, IL-10, or TGF- β , were minor or not significant. Furthermore, transfer of activated eosinophils to SCID recipients led to a much weaker but significant induction in IFN- γ expression (Fig 3, A) that persisted at least for 7 days (see Fig E3, B). This low production is in agreement with the fact that lymphocytes are a major source of IFN- γ . Expression of IL-6, IL-10, and TGF- β was also induced in SCID recipients.

Because IFN- γ was detected in eosinophil-transferred SCID mice and because several myeloid cell types,²¹ including human eosinophils,⁴ produce IFN- γ , we reasoned that such a nonlymphocytic source was essential to the development of lung inflammation in our model. IFN- γ expression was thus examined by means of intracellular flow cytometry in splenic eosinophils from IL-5 Tg mice. IFN- γ was significantly expressed in activated eosinophils (9.6%) compared with naive eosinophils (4.1%). By contrast, no staining was observed in eosinophils from IL-5 Tg mice on an IFN- γ -deficient background (IFN- $\gamma^{-/-}$ eosinophils; Fig 3, B). In addition, we were also able to detect IFN- γ mRNA expression on highly purified eosinophils despite very low expression levels compared with those on T cells (see Fig E5, B, in this article's Online Repository at www.jacionline.org). Therefore eosinophils can in part account for IFN- γ production detected in SCID mice.

IFN- γ production by eosinophils is essential for lung inflammation

To determine the contribution to lung inflammation of eosinophil-produced IFN- γ *in vivo*, we used 3 complementary approaches. First, we neutralized endogenously produced IFN- γ in recipients by means of injection of a neutralizing anti-IFN- γ antibody (or an isotype control) before the transfer of activated eosinophils. Second, we used IFN- $\gamma^{-/-}$ mice as recipients. Third, we adoptively transferred 4×10^7 IFN- $\gamma^{+/+}$ or IFN- $\gamma^{-/-}$ activated eosinophils.

In IFN- $\gamma^{+/+}$ recipients anti-IFN- γ treatment significantly but partially inhibited changes in resistance and compliance induced by transfer of IFN- $\gamma^{+/+}$ eosinophils (Fig 4, A and B), suggesting that endogenous IFN- γ production contributes but is not essential

to AHR in our experimental model. Furthermore, transfer of IFN- $\gamma^{+/+}$ activated eosinophils to IFN- $\gamma^{-/-}$ mice induced a significant increase (346% over baseline) in resistance and a decrease (52% under baseline) in compliance compared with PBS-treated recipients (Fig 4, C and D), confirming that endogenous IFN- γ in recipients is not required for AHR induction. Finally, transfer of IFN- $\gamma^{-/-}$ activated eosinophils completely failed to induce AHR in IFN- $\gamma^{-/-}$ (Fig 4, C and D), IFN- $\gamma^{+/+}$ (see Fig E6, A and B, in this article's Online Repository at www.jacionline.org), and SCID mice (Fig E6, C and D). In addition to their effect on AHR, activated IFN- $\gamma^{+/+}$ eosinophils also induced recruitment of inflammatory cells and fibrosis within lung tissue in IFN- $\gamma^{-/-}$ mice, whereas IFN- $\gamma^{-/-}$ eosinophils only induced a minor inflammatory cell recruitment (Fig 4, E) and collagen deposition (Fig 4, F). Furthermore, compared with IFN- $\gamma^{+/+}$ eosinophils, IFN- $\gamma^{-/-}$ eosinophils induced a weaker but still significant recruitment of inflammatory cell, eosinophil, and macrophage recruitment in airway lumen (Fig 4, G). Taken together, these data show that in this model IFN- γ production by eosinophils is essential for the induction of AHR, exerts a major effect on fibrosis and lung cellular inflammation, and contributes to cellular recruitment in BALF.

Autocrine stimulation of eosinophils by IFN- γ

Because human eosinophils express and can be activated through the IFN- γ receptor,²² we considered that the critical effect of eosinophil-derived IFN- γ on lung inflammation might be in part due to an autocrine stimulation of eosinophils themselves. We thus examined expression of both the α and β chains of the IFN- γ receptor²³ on eosinophils from IL-5 Tg mice. Surface expression levels were low for both chains. However, expression of IFN- γ receptor 1 (IFN- γ R1) on activated eosinophils (7.3%) was significantly higher than that on naive eosinophils (2.6%). Moreover, an important pool of intracellular chains was detected (Fig 5, A). Expression of both the IFN- γ R1 and IFN- γ R2 chains was also detected by means of PCR in eosinophils from IL-5 Tg and was found to be 8 times lower than expression on CD4⁺ lymphocytes from the same animals (data not shown). Furthermore, preincubation of IFN- $\gamma^{+/+}$ eosinophils with a blocking anti-IFN- γ R1 antibody before transfer into naive IFN- $\gamma^{-/-}$ recipient significantly decreased eosinophil and macrophage recruitment in BALF (see Fig E7, A, in this article's Online Repository at www.jacionline.org) and totally abrogated changes in resistance and compliance observed on treatment with control isotype antibody (see Fig E7, B and C).

To confirm the direct effect of IFN- γ on eosinophil function, we stimulated IFN- $\gamma^{+/+}$ and IFN- $\gamma^{-/-}$ eosinophils *in vitro* with increasing concentrations of IFN- γ and found a comparable dose-dependent increase in EPO production (Fig 5, B). Moreover, overnight culture in the presence of exogenous IFN- γ also promoted comparable survival of IFN- $\gamma^{+/+}$ and IFN- $\gamma^{-/-}$ eosinophils, although the maximal effect was obtained for 100-fold lower concentrations than for induction of EPO release (Fig 5, C). Such an effect was abrogated on preincubation with

FIG 5. IFN- γ modulates eosinophil function. **A**, Surface and intracellular expression of IFN- γ R1 and IFN- γ R2 on naive or activated IFN- $\gamma^{+/+}$ or IFN- $\gamma^{-/-}$ eosinophils ($n = 3$ -5 per group). **B-D**, The effect of IFN- γ on eosinophil function *in vitro*: **Fig 5, B**, EPO release ($n = 3$ -8 per group); **Fig 5, C**, eosinophil survival ($n = 4$ -7 per group); **Fig 5, D**, Eotaxin-induced chemotaxis ($n = 4$ -8 per group). Statistically different ($P < .05$) from *PBS-treated eosinophils and §isotype IgG-treated or IFN- $\gamma^{+/+}$ transferred eosinophils ($P < .05$).

an anti-IFN- γ receptor antibody (Fig 5, B and C). Interestingly, a high concentration of exogenous IFN- γ significantly decreased eosinophil survival and migratory ability (Fig 5, C and D). Furthermore, eotaxin-induced chemotaxis of IFN- $\gamma^{-/-}$ eosinophils was lower than that from their IFN- $\gamma^{+/+}$ counterparts. IFN- γ potentiated chemotaxis of both IFN- $\gamma^{+/+}$ and IFN- $\gamma^{-/-}$ eosinophils but failed to restore normal migration in the later ones (Fig 5, D). Thus IFN- γ deficiency affects selective activation parameters rather than leading to a general functional defect.

DISCUSSION

We investigated the pulmonary response induced by adoptive transfer of eosinophils to elucidate the fundamental role of these cells *in vivo*. We observed that the transfer of 2×10^7 highly purified eosinophils from IL-5 Tg mice activated by OVA *in vivo* could remarkably induce AHR and lung inflammation in naive recipient BALB/c mice. By contrast, Shen et al⁷ previously reported that adoptive transfer of 4×10^7 eosinophils to naive IL-5^{-/-} recipient mice did not affect AHR, whereas donor eosinophils promoted development of an allergic pulmonary reaction in OVA-sensitized/challenged recipient mice unless they were depleted of CD4⁺ cells. Despite apparent discrepancies, our results, together with the data of Shen et al, show that eosinophils need to be activated to induce significant AHR. Furthermore, in both instances, lymphocytes are present during eosinophil activation, which takes place in donor mice in our model and in recipient animals in the Shen et al model. In SCID recipient mice IL-5 might still be produced by nonlymphocytic cells, as reported for human airway epithelial cells,²⁴ in quantities sufficient to allow the maintenance of eosinophil activation, survival, or both in the absence of lymphocytes. By contrast, in the Shen et al model,⁷ where IL-5 is completely lacking in recipient mice during eosinophil activation, synthesis by lymphocytes of other eosinophilic cytokines and chemokines is probably essential for eosinophil activation, survival, or both. As additional differences between the models, we transferred spleen eosinophils from BALB/c IL-5 Tg mice, whereas Shen et al used blood eosinophils from C57BL/6 IL-5 Tg mice. Furthermore, our results obtained with SCID recipient mice show that activated eosinophils also promoted, in a dose-dependent manner, AHR and lung inflammation in the absence of lymphocytes. Even though this latter cell type exerts a major contribution to the intensity and persistence of the lung inflammatory reaction, it thus appears that in addition to their ability to attract T_H2 lymphocytes,^{8,9} eosinophils are able to play an intrinsic role at an early stage of the lung inflammatory reaction. In our model, eosinophil-derived IFN- γ is essential to the development of AHR not only in IFN- γ -deficient animals but also in fully IFN- γ -proficient BALB/c mice and lymphocyte-deficient SCID animals. T_H1 responses, in particular IFN- γ , are usually considered to inhibit development of allergic asthma, T_H2-associated responses, and eosinophilia.²⁵⁻²⁹ However, few studies in human subjects and mice have reported on the positive contribution of T_H1 lymphocytes and IFN- γ to allergic lung inflammation.³⁰⁻³³ These discrepancies might be in part attributed, as pointed out by some of these authors, to differences in experimental models (dose, timing, and means of IFN- γ or anti-IFN- γ administration; use of IFN- γ - or IFN- γ receptor-deficient animals and genetic background) but might also reflect a dual role for IFN- γ , as recently suggested for autoimmune diseases.³⁴

Nevertheless, the positive or negative effects observed on airway inflammation were considered to target or arise from lymphocytes. In our experimental conditions eosinophils not only induce IFN- γ production in inflamed lung tissue but are themselves both the source and target of IFN- γ , as it is the case for human eosinophils. Indeed, on the one hand we previously demonstrated that human eosinophil activation by CD28 led to IFN- γ production.⁴ On the other hand, human eosinophils express IFN- γ R1, and its activation by IFN- γ upregulates CD69 and eosinophil cationic protein expression²² and prolongs survival.^{35,36} We found that IFN- γ R1 surface expression was upregulated on activated murine eosinophils, whereas IFN- γ R2 was only detected intracellularly. Low IFN- γ R2 surface expression on T cells has been shown to induce proliferation and to reduce signal transducer and activator of transcription 1 and interferon regulatory factor 1, the activation of which induces apoptosis.³⁷ *In vitro* IFN- γ acted on eosinophils either as an activator at low concentrations or as an inhibitor at high concentrations. Such a biphasic effect on eosinophils could in part explain earlier work reporting that exogenous IFN- γ attenuated the allergic response, whereas endogenous IFN- γ contributed to an optimal response during the development of allergic asthma.²⁷

Our data establish that in addition to their classical cytotoxic activity and their recently identified role in the local recruitment of T_H2 lymphocytes at inflammatory sites, eosinophils significantly contribute to lung inflammation, even in the absence of lymphocytes, through IFN- γ production and autocrine stimulation. This unexpected finding further underlines that caution must be exerted when considering alterations of T_H1/T_H2 balance by antibodies such as anti-IL-5 or IFN- γ as therapeutic tools.

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Clinical Implications: Use of IFN- γ to inhibit T_H2-associated pathologies has been considered. Our results suggest that eosinophil-derived IFN- γ is an important mediator of lung inflammation. Caution is thus required in the use of T_H1/T_H2 balance modulators.

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METHODS

Flow cytometry

Unless specified otherwise, staining was performed with antibodies from BD PharMingen for 30 minutes at 4°C on 5×10^5 cells in 100 μ L after blocking nonspecific binding to Fc γ R with anti-CD16/CD32 (2.4G2). Eosinophils were labeled with Ly-6G mAbs conjugated to phycoerythrin and biotinylated anti-IFN- γ R1 or purified anti-IFN- γ R2 followed by anti-hamster IgG-biotin and streptavidin-allophycocyanin.

For intracellular staining, fixation and permeabilization were performed with the Cytofix/Cytoperm Fixation/Permeabilization kit (BD PharMingen), according to the manufacturer's instructions. For detection of intracellular IFN- γ , eosinophils were preincubated for 6 hours at 37°C with the protein transport inhibitor BD GolgiPlug containing brefeldin A in RPMI 1640 with 10% mouse serum followed by fixation, permeabilization, and staining with allophycocyanin-conjugated anti-IFN- γ . Cells were analyzed on a FACSCalibur (Becton Dickinson) flow cytometer by using Cell Quest software.

Cytokines were measured in BALF by using the Cytokine Beads Array (BD PharMingen), according to the manufacturer's instructions.

Histology

Left lung samples were fixed with Immunohistofix (Intertiles, Bruxelles, Belgium). Embedding was performed in Immunohistowax (Intertiles), and 5- μ m sections were obtained after dehydration. Staining was performed with Matrius-Scarlet-Blue^{E1} for morphology or periodic acid-Schiff^{E2} for detection of mucopolysaccharide accumulation. The mucus index was calculated and analyzed by using Image J software as follows:

$$\frac{(\text{Average intensity of epithelial periodic acid - Schiff staining}) \times (\text{Area of epithelial periodic acid - Schiff staining})}{(\text{Total area of conducting airway epithelium})}^{E2}$$

Quantification of collagen and fibrogenic factors

Collagen deposition in the total left lung was determined by using the Sircol Collagen Assay Kit (Biocolor, Carrickfergus, United Kingdom), according to the manufacturer's instructions.^{E2} Matrix metalloproteinase 9, tissue inhibitor of metalloproteinase 1, vascular endothelial growth factor, and TGF- β were quantified by using ELISA kits (R&D Systems), according to the manufacturer's recommendations.

Quantitative RT-PCR

RNA was isolated from total right lung or sorted eosinophils by using the Guanidium/Cesium Chloride method.^{E3} Reverse transcription was performed with 1 μ g of RNA by using Superscript reverse transcriptase (Invitrogen, Cergy Pontoise, France), according to the manufacturer's instructions. cDNA was amplified by means of PCR in triplicate for 40 cycles in Power SYBR Green PCR Master Mix (Molecular Probes, Leiden, The Netherlands) on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Courtaboeuf, France). Primer pairs listed in Table E1 were used for amplification. PCR amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was performed to control for sample loading and to allow normalization between samples. Results were expressed as the mean fold increase compared with the expression level in PBS-transferred recipient mice.

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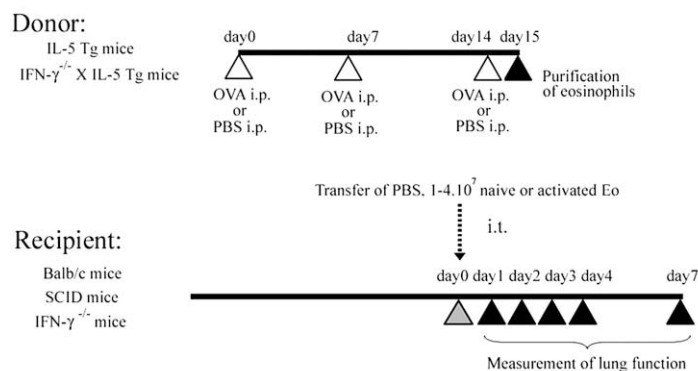
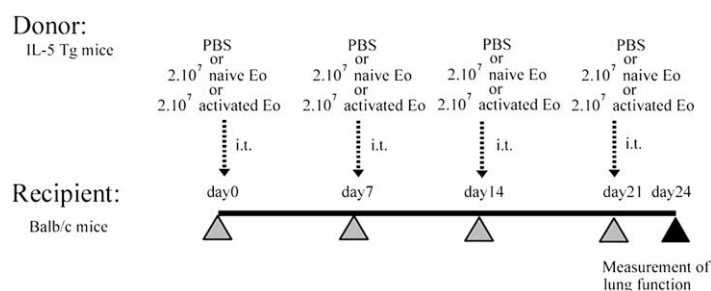
A Short term model; transfer of eosinophils x1**B Long term model; transfer of eosinophils x4**

FIG E1. Experimental design for eosinophil transfer. **A**, Short-term protocol. Eosinophils are purified from IFN- $\gamma^{+/+}$ X IL-5 Tg or IFN- $\gamma^{-/-}$ X IL-5 Tg mice after 3 intraperitoneal injections of OVA (*activated Eo*) or PBS (*naive Eo*) 1 week apart and killed 24 hours after the last injection. Eosinophils ($1-4 \times 10^7$) were transferred into naive recipient mice by means of intratracheal (*i.t.*) injection (control animals received PBS). Animals were killed 1, 2, 3, 4, or 7 days later. *i.p.*, Intraperitoneal. **B**, Long-term protocol. Naive recipients received 4 intratracheal injections of 2×10^7 naive or activated eosinophils 1 week apart (control animals received PBS).

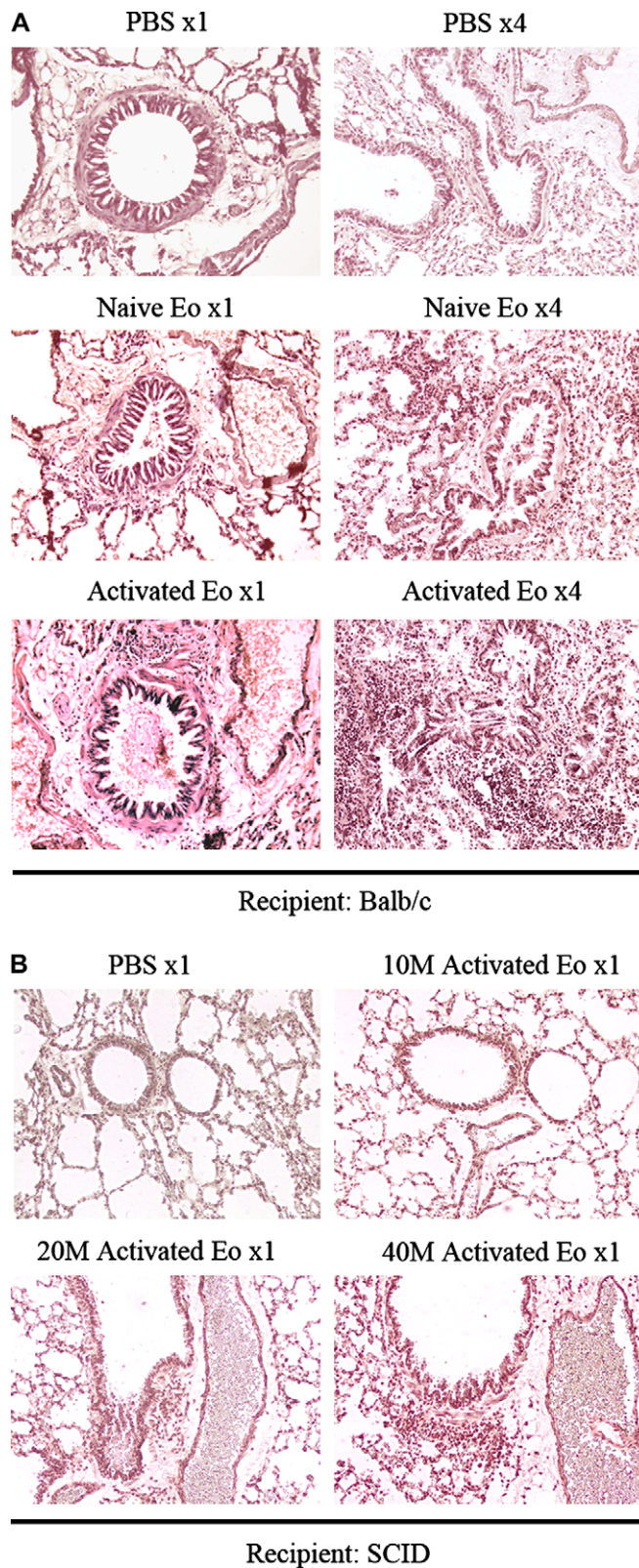


FIG E2. Mucus accumulation after eosinophil transfer in BALB/c and SCID mice: wild-type recipients (**A**) or SCID recipients (**B**). Periodic acid-Schiff staining was used (original magnification $\times 100$; $n = 3-5$ per group). *Statistically different ($P < .05$) from PBS-treated recipient mice.

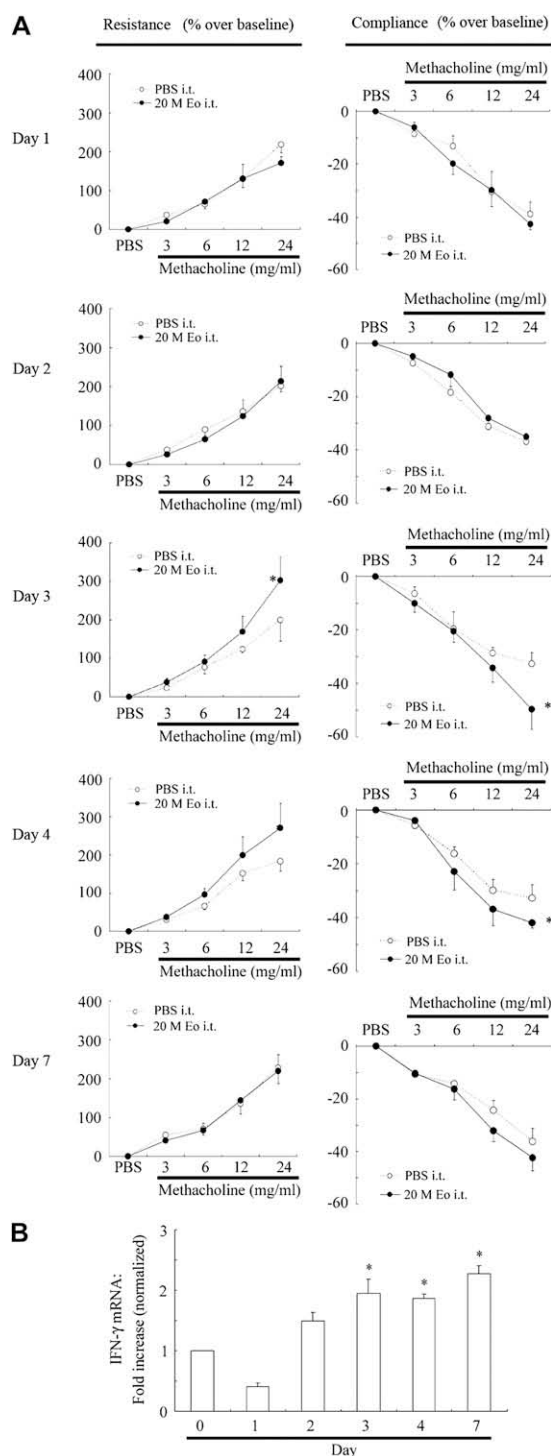


FIG E3. Kinetics of eosinophil-induced lung inflammation in SCID mice. Resistance, compliance (**A**), and lung IFN- γ mRNA content (**B**) were measured at the indicated time points after transfer of 2×10^7 activated eosinophils ($n = 3-4$ per group). *Statistically different ($P < .05$) from PBS-treated recipient mice. *i.t.*, Intratracheal.

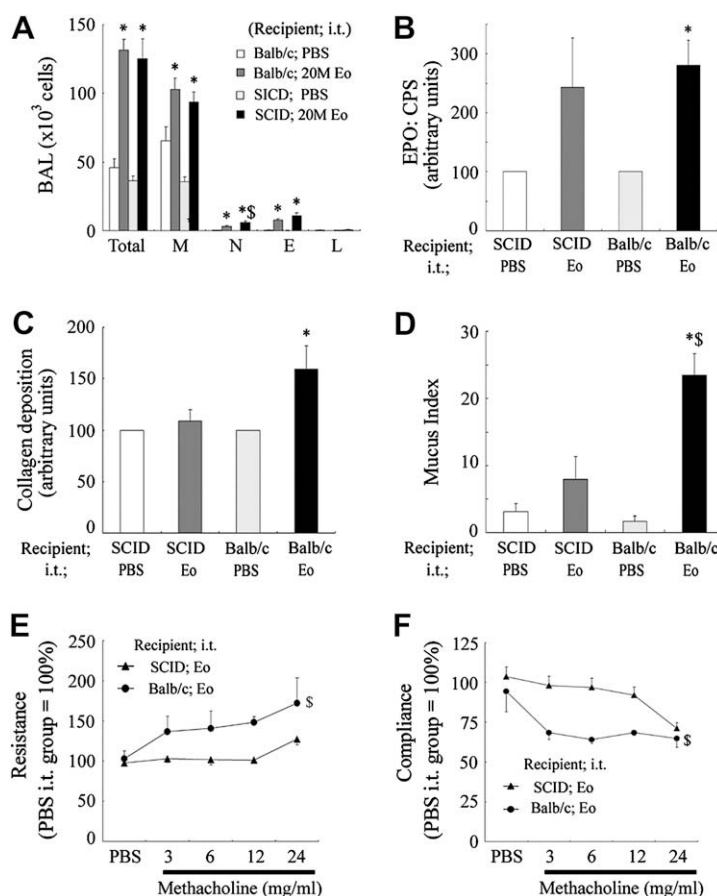


FIG E4. Comparison of eosinophil-induced lung inflammation in BALB/c and SCID mice. PBS or 2×10^7 activated eosinophils (Eo) were transferred once to BALB/c mice and SCID animals that were killed 72 hours later. **A**, BALF cellularity: macrophages (M), neutrophils (N), eosinophils (E), and lymphocytes (L; $n = 6-9$ per group). **B**, EPO activity in BALF ($n = 3-7$ per group). **C**, Collagen deposition ($n = 9-23$ per group). **D**, Mucus index ($n = 3-6$ per group). **E** and **F**, Airway resistance (Fig E4, E) and compliance (Fig E4, F; $n = 3-5$ per group). Values for the PBS group are set at 100%. Open and solid symbols, 1 and 4 transfers, respectively. Statistically different ($P < .05$) from *PBS-treated recipient mice and § SCID recipient mice.

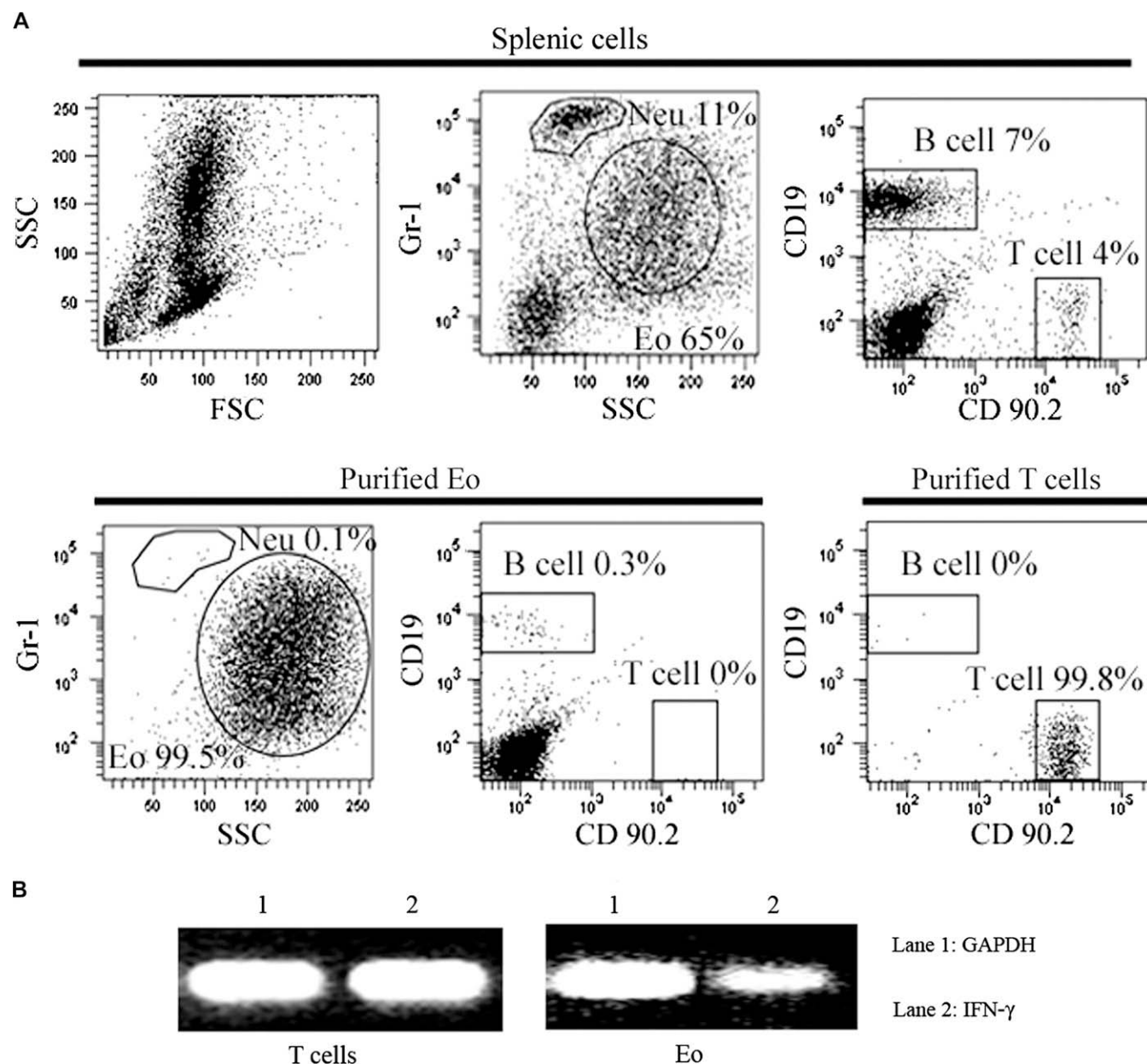


FIG E5. IFN- γ mRNA expression in highly purified eosinophils (Eo) from IL-5 Tg mice. **A**, Flow cytometric analysis of cell populations in the spleen (upper row). Analysis of purified eosinophil and T-cell populations after cell sorting (lower row). SSC, Side scatter; FSC, forward scatter. **B**, RT-PCR analysis of IFN- γ in eosinophils and T lymphocytes. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

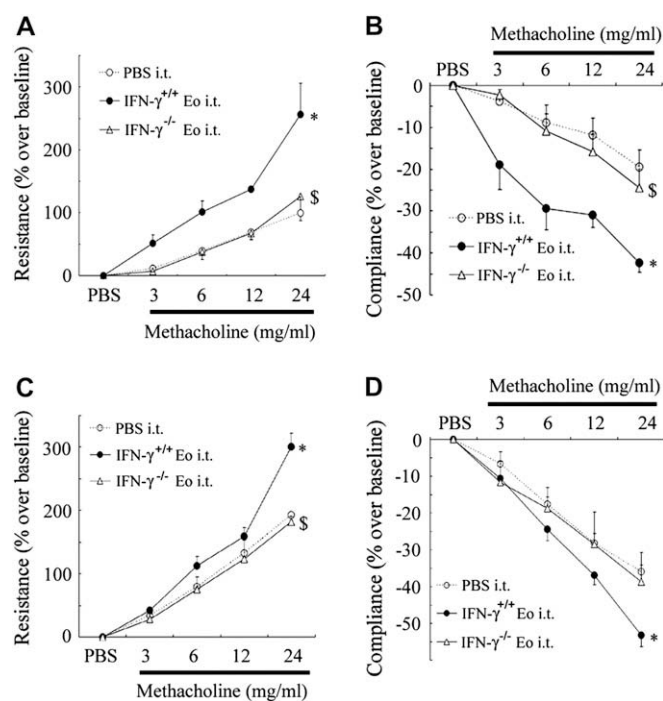


FIG E6. Eosinophil-derived IFN- γ is essential for eosinophil-induced lung inflammation in BALB/c and SCID mice. **A-D**, AHR and inflammation 72 hours after transfer of 2×10^7 (Fig E6, A and B) or 4×10^7 (Fig E6, C and D) IFN- $\gamma^{+/+}$ or IFN- $\gamma^{-/-}$ activated eosinophils in naive BALB/c (Fig E6, A and B) and SCID (Fig E6, C and D) recipient mice. Airway resistance (Fig E6, A and C) and compliance (Fig E6, B and D) after PBS, IFN- $\gamma^{+/+}$, and IFN- $\gamma^{-/-}$ activated eosinophil administration ($n = 3-7$ per group) is shown. Statistically different ($P < .05$) from *PBS-treated recipient mice and §IFN- $\gamma^{+/+}$ transferred eosinophils. Eo, Eosinophils; i.t., intratracheal.

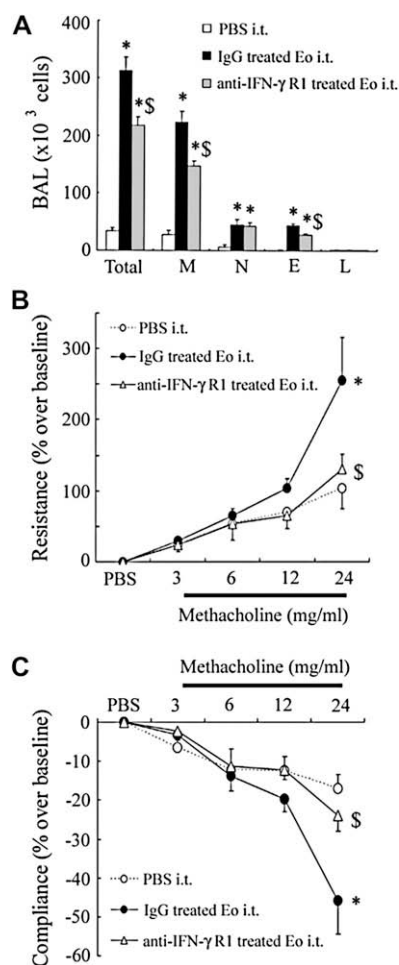


FIG E7. Autocrine action of IFN- γ on eosinophil-induced lung inflammation. Purified activated eosinophils (Eo ; 4×10^7 cells) were pretreated with anti-IFN- γ R1 antibody or isotype IgG for 1 hour and transferred to naive IFN- $\gamma^{-/-}$ recipient mice, which were killed 72 hours later. **A**, BAL cellularity: macrophages (M), neutrophils (N), eosinophils (E), and lymphocytes (L ; $n = 4-8$ per group). **B** and **C**, Airway resistance (Fig E7, **B**) and compliance (Fig E7, **C**; $n = 4-8$ per group). Statistically different ($P < .05$) from *PBS-treated recipient mice and §isotype IgG-treated eosinophils. *i.t.*, Intratracheal.

TABLE E1. Primers used for real-time PCR

Primers	Forward	Reverse
TGF- β	5' TAC TCT GGA GAC GGT TTG CCA 3'	5' CAT GAA GAA AGT CTC GCC CG 3'
IL-6	5' CCA CGG CCT TCC CTA CTT CA 3'	5' CCA CGA TTT CCC AGA GAA CAT G 3'
IL-9	5' GAC CAG CTG CTT GTG TCT CTC C 3'	5' CCG ATG GAA AAC AGG CAA GA 3'
IL-10	5' TTC ATG GCC TTG TAG ACA CC 3'	5' TGA ATT CCC TGG GTG AGA AG 3'
IFN- γ	5' ACC CTG TCG TAT GCT GGG AA 3'	5' GTT GGT GCA GGA ATC AGT CCA 3'
T-bet	5' CCCACAAGCCATTACAGGATGT 3'	5' TGCCTTCTGCCTTTCCACAC 3'
IL-4	5' GCA TGG AGT TTT CCC ATG TT 3'	5' AGA TGG ATG TGC CAA ACG TC 3'
IL-13	5' GGA ATC CAG GGC TAC ACA GA 3'	5' AGG AGC TGA GCA ACA TCA CA 3'
GATA-3	5' GCCTGCGGACTCTACCATAA 3'	5' CATTAGCGTTCCTCCTCCAG 3'
IFN- γ R1 (α chain)	5' ATGATTCTGCTGGTGGTCCT 3'	5' CAGCATACGACAGGGTTCAA 3'
IFN- γ R2 (β chain)	5' GCTGGAGCCATTTCAACACT 3'	5' ACATCAAAGGGAGGGGAGAA 3'
GAPDH	5' TGC CCA GAA CAT CAT CCC TG 3'	5' TCA GAT CCA CGA CGG ACA CA 3'

T-bet, T-box transcription factor; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.