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# IFN- $\gamma$ Acts on the Airway Epithelium To Inhibit Local and Systemic Pathology in Allergic Airway Disease

Charlotte Mitchell,<sup>\*1</sup> Karin Provost,<sup>\*1</sup> Naiqian Niu,<sup>\*</sup> Robert Homer,<sup>†</sup> and Lauren Cohn<sup>\*</sup>

**Inhibiting allergic airway inflammation is the goal of therapy in persistent asthma. Administration of medication via the airways delivers drug directly to the site of inflammation and avoids systemic side effects but often fails to modulate systemic features of asthma. We have shown that Th1 cells, through production of IFN- $\gamma$ , inhibit many Th2-induced effector functions that promote disease. Using a newly generated mouse that expresses IFN- $\gamma$ R only on airway epithelial cells, we show that the airway epithelium controls a range of pathological responses in asthma. IFN- $\gamma$  acting only through the airway epithelium inhibits mucus, chitinases, and eosinophilia, independent of Th2 cell activation. IFN- $\gamma$  signaling through the airway epithelium inhibits eosinophil generation in the bone marrow, indicating that signals on the airway mucosal surface can regulate distant functions to inhibit disease. IFN- $\gamma$  actions through the airway epithelium will limit airway obstruction and inflammation and may be therapeutic in refractory asthma. *The Journal of Immunology*, 2011, 187: 3815–3820.**

In asthma, CD4 Th2 cells are present in the airways during acute exacerbations of disease and remain active even when symptoms are quiescent (1, 2). Animal studies show that CD4 Th2 cells are required for development of Ag-induced allergic airway inflammation, and overexpression of the Th2 cytokines, IL-4, IL-5, and IL-13, results in features associated with chronic asthma (3–5). Th2 cytokines promote airway eosinophilia and increased production of mucus and chitinases leading to disease. Airway epithelial cell mucus hypersecretion is IL-13 dependent and contributes significantly to airway obstruction and death during acute exacerbations of asthma (6, 7). Airway eosinophilia correlates both with disease activity and severity (8). IL-5 and IL-13 stimulate bone marrow (BM) production of eosinophils and recruitment of eosinophils to the respiratory tract (9). Chitinases, produced by airway epithelial cells and macrophages, are induced by IL-4 and IL-13 in the airways of asthmatics, are associated with disease severity, and promote inflammation (10). These important Th2-dependent effects indicate asthma's scope, with both local and systemic contributions to disease development and activity. Inhibition of Th2 cell function and/or blockade of Th2 effector responses are the goals of therapy in asthma. Still, in many cases, standard therapies do not effectively inhibit disease, or their long-term use is limited because of adverse systemic effects.

Th1 cells have also been identified in the airways of asthmatics, and their function has been controversial (11, 12). Th1 cells alone do not produce any of the characteristic features of asthma in mice, and some studies showed that mixed populations of Th1 and Th2 cells inhibit asthmatic responses through production of IFN- $\gamma$  (13–15). In other studies, though, mixed populations of Th1 and Th2 cells enhanced inflammation (16, 17). Understanding the various mechanisms of Th1 cell and IFN- $\gamma$  regulation in allergic airway disease may define pathways to target for new asthma therapies.

IFN- $\gamma$  binds to its receptor and activates hundreds of genes leading to proinflammatory effects by increasing Ag processing and presentation as well as anti-inflammatory effects because of its apoptotic and antiproliferative functions. IFN- $\gamma$  inhibits Th2 cell function (18). In models of asthma, IFN- $\gamma$  reduces recruitment of lymphocytes and eosinophils and inhibits airways hyperresponsiveness and mucus but promotes airway neutrophils and overall lung inflammation (14–17, 19, 20). IFN- $\gamma$  may stimulate eosinophil activation, longevity, or apoptosis (21, 22). Since the dose, sites of action, and the cell-specific responses will influence these effects of IFN- $\gamma$ , we investigated how IFN- $\gamma$  would influence Th2-induced allergic airway disease if IFN- $\gamma$  effects were directed to the airways. In this study, we use a novel transgenic mouse that expresses IFN- $\gamma$ R only on airway epithelial cells and show the diverse airway epithelial-specific effects of IFN- $\gamma$ .

<sup>\*</sup>Section of Pulmonary and Critical Care, Yale University School of Medicine, New Haven, CT 06520; and <sup>†</sup>Department of Pathology, Yale University School of Medicine, New Haven, CT 06520

<sup>1</sup>C.M. and K.P. contributed equally to the manuscript.

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Address correspondence and reprint requests to Dr. Lauren Cohn, Section of Pulmonary and Critical Care, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208057, New Haven, CT 06520-8057. E-mail address: lauren.cohn@yale.edu

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; CC, rat Clara cell; HMI, histological mucus index; PAS, periodic acid-Schiff; qPCR, quantitative PCR; WT, wild-type.

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## Materials and Methods

### Mice

OT-II mice, which are transgenic for the TCR recognizing OVA peptide<sup>323–339</sup>, were bred in our facilities and backcrossed to B6.PL-*Thy1a*/Cy (The Jackson Laboratory, Bar Harbor, ME). OT-II<sup>Thy1.1</sup> and OT-II<sup>Thy1.1/1.2</sup> mice were used for the generation of Th1 and Th2 cells. C57BL/6 mice and *ifngr1*<sup>-/-</sup> mice (The Jackson Laboratory or National Cancer Institute, Frederick, MD) were used as recipients of cell transfers at 6–8 wk of age. IL-5 transgenic mice were used as donors for eosinophil migration assays (23). All experimental protocols were approved by the Yale Animal Care and Use Committee.

### Generation and confirmation of epi-IFN- $\gamma$ R mice

To generate “epi-IFN- $\gamma$ R” mice, a construct was generated containing the murine IFN- $\gamma$ R1 cDNA (provided by Dr. R. Schreiber, Washington University, St. Louis, MO) with a 39-bp human c-myc tag inserted after the

signal peptide. This was cloned into a construct containing the rat Clara cell (CC)10 promoter and human growth hormone intronic and polyadenylation sequences (donated by Dr. P. Ray, University of Pittsburgh, Pittsburgh, PA) as described previously (24). After the fidelity of the junction areas and *ifngr1* cDNA were confirmed by sequencing, the EcoRV and BglII fragment containing CC10 promoter, c-myc, mouse *ifngr1* cDNA, and hGH 3' untranslated region was digested and isolated from the vector by electrophoresis. The purified DNA was microinjected into C57BL/6 pronuclei. Transgene-positive mice were identified by PCR using primers for c-myc (5'-ATGGCATCAATGCAGAAGCTGATCTCA, 3'-TCAGAAATGTTGGTGCAGGA). Transgene-positive mice were crossed to *ifngr1*<sup>-/-</sup> mice to generate epi-IFN- $\gamma$ R mice that express IFN- $\gamma$ R on CC10-expressing cells only. To confirm lung-specific expression of IFN- $\gamma$ R1, lung and spleen RNA were isolated, and quantitative PCR (qPCR) was performed as detailed below. To determine response to IFN- $\gamma$ , C57BL/6, *ifngr1*<sup>-/-</sup>, and epi-IFN- $\gamma$  mice were given 2  $\mu$ g IFN- $\gamma$  (BD Biosciences, Franklin Lakes, NJ) intranasally once a day for 3 d and sacrificed. Lungs were excised and treated with dispase to liberate epithelial cells. Lung cell suspensions were stained with Abs to CD45, cytokeratin, MHC class I, and MHC class II (BD Biosciences) and analyzed by flow cytometry. Immunofluorescence with anti-CC10 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) was performed on cytospins of lung cell suspension.

#### Generation of Th1 or Th2 cells and Th cell transfer

Generation of Th1 or Th2 cells from CD4 T cells from OT-II mice was described previously (25). After 4 d of culture, cells were transferred into recipient mice. A total of 1–2.5  $\times 10^6$  Th1 or Th2 cells, or both Th1+Th2 cells (1–2.5  $\times 10^6$  of each), were injected i.v., and 1 d after transfer of cells, mice were challenged with inhaled 1% OVA in PBS for 20 min daily for a total of 7 d over a period of 9 d and sacrificed on day 10. At the time of transfer, an aliquot of Th1- or Th2-like cells was retained for restimulation, and supernatants were collected at 24 h for determination of IFN- $\gamma$ , IL-4, and IL-5 levels from cell supernatants by ELISA (Endogen, Cambridge, MA). Th2 cell populations used in these experiments typically produced IL-4 (4–20 ng/ml), IL-5 (2–12 ng/ml), and IFN- $\gamma$  (<150 ng/ml). Th1 cell populations used produced IFN- $\gamma$  (230–1200 ng/ml) and undetectable IL-4 and IL-5.

#### Cell isolation from airway, lung, and BM

Bronchoalveolar lavage (BAL) and isolation of lung leukocytes was performed as described previously (25). BM was isolated from one femur of each mouse. Cells were counted, and differentials were performed on cytospin preparations stained with Diff-Quik (Baxter Healthcare, Miami, FL). At the time of sacrifice, flow cytometry was performed on BAL and lung specimens to identify the transferred cells using Abs to CD4, Thy1.1, and Thy1.2.

#### Cytokine, chemotaxis assays, and chitinase assays

Multiplexed fluorescent bead analysis (eotaxin, Mig, inflammatory protein-10, IL-4, IL-5, and IFN- $\gamma$ ; Millipore, Billerica, MA) and ELISA (IL-13; Invitrogen, Carlsbad, CA) were used to quantify levels in BAL fluid (BALF). For eosinophil chemotaxis assay, eosinophils were isolated from the blood of NJ1628 mice, as described previously (26). A total of 7.5  $\times 10^5$  cells (of which  $\geq 95\%$  were eosinophils) were placed in the upper chamber of ChemoTx System (NeuroProbe, Gaithersburg, MD) with BALF or control medium in the lower chamber, and the plate was incubated at 37° for 90 min. From duplicate samples, the mean number of eosinophils that migrated into the lower chamber is reported. Chitinase activity in cell-free BALF was assayed using the fluorogenic substrate 4MU-GlcNAc2 (10) and reported as fluorescence units.

#### qPCR

Lung or spleen RNA was isolated, cDNA was synthesized, and qPCR was performed using the SYBR Green System (Applied Biosystems, Carlsbad, CA). Primers were as follows: IFN- $\gamma$ R (forward, 5'-CTCCAATCCGACACAAAGACG-3'; reverse, 5'-TGATAGGCGGTGAGGCTACAAG-3') and  $\beta$ -actin (forward, 5'-GTCCACACCCGCCACAGTTCG-3'; reverse, 5'-GACCCATTCCCACCATCACCCCT-3'); and *Muc5AC* (forward, 5'-ATCTTCAGGACCCCTGCTCGCT-3'; reverse, 5'-ACATGCAGTTCGAGAAGAAGGTGC-3'), *AMCase* (forward, 5'-ACAAGCATCTCTCACTGTCCTGGT-3'; reverse, 5'-TGGATGTTGGAAATCCCACCAGCT-3') and  $\beta$ -actin (forward, 5'-GTCCACACCCGCCACAGTTCG-3'; reverse, 5'-GACCCATTCCCACCATCACCCCT-3'). For each set of primers, validation experiments showed linear dependency of the threshold cycle values at different RNA concentrations. The data were analyzed after genes were normalized to  $\beta$ -actin, and *p* values were obtained using Data Assist software (Applied Biosystems).

#### Histological mucus index

Formalin-fixed, paraffin-embedded lungs were stained with PAS. A histological mucus index (HMI) was calculated using periodic acid-Schiff (PAS)-stained lung sections as described previously (13). This index is the equivalent to the linear percent of epithelium positive for mucus and was calculated for each mouse lung by a blinded investigator, and then, the mean of the HMI was calculated for each experimental group.

#### Data analysis

Data are reported as mean  $\pm$  SE. Statistical significance was determined by unpaired Student *t* test using the Bonferroni correction for multiple comparisons.

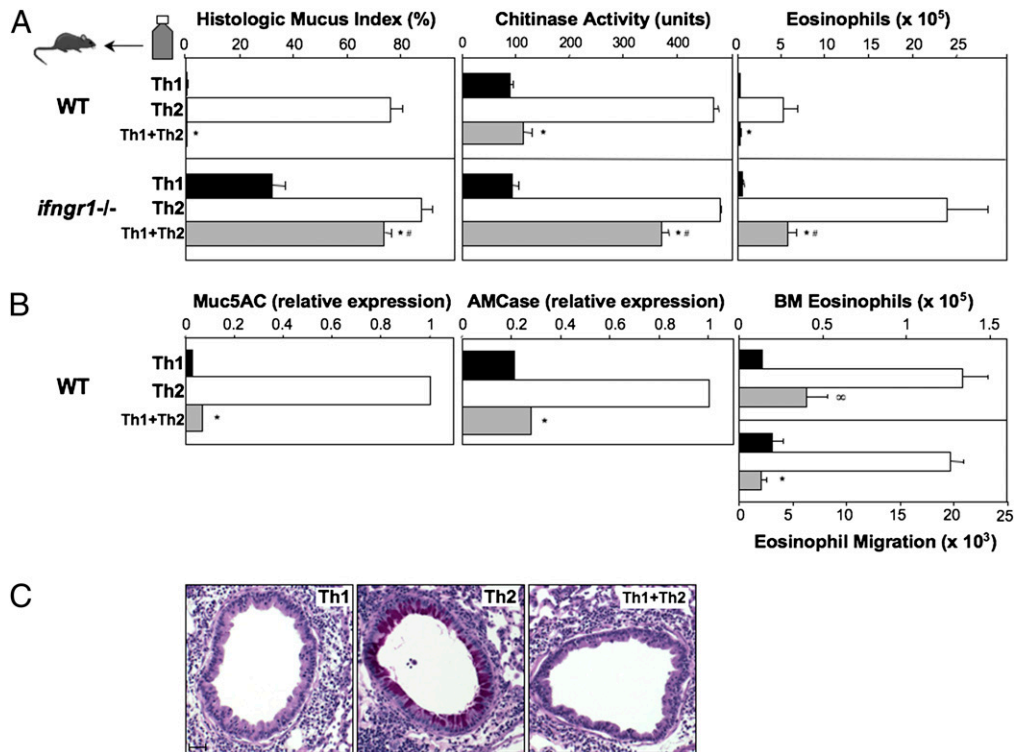
## Results

### IFN- $\gamma$ inhibits Th2-induced mucus, chitinases, and eosinophilia

Our previous studies established that Th1 cells, through production of IFN- $\gamma$ , inhibit Th2-induced effector functions (14). Those studies, in BALB/c mice, revealed that IFN- $\gamma$  inhibited Th2 effects in the lung. In the current studies, using C57BL/6 mice, we investigate the specific mechanisms by which Th1 cells and IFN- $\gamma$  inhibit critical pathogenic features of allergic airway inflammation, focusing on mucus hyperproduction, increased chitinase activity, and airway eosinophilia. To study how Th1 cells inhibit Th2 cell effects, we cotransferred OVA-specific OT-II Th1 and Th2 cells into C57BL/6 recipient mice. After adoptive transfer, mice were exposed to inhaled OVA over 9 d, and both Th1 and Th2 cells were recruited to the lung. Th1 cells inhibited Th2 cell-induced mucus staining, chitinase activity, and eosinophilia in the airways (Fig. 1A) and lung (data not shown). Th1 and Th2 cells were distinguished by congenic markers for Thy<sup>1.1</sup> or Thy<sup>1.1/1.2</sup>. Th2 cytokines and the number of Th2 cells were comparable in mice that received either transfer of Th1+Th2 cells or Th2 cells alone (Supplemental Fig. 1). Therefore, Th1 cells inhibit these Th2 cell effects in the presence of activated Th2 cells in the respiratory tract. Although it is well known that IFN- $\gamma$  can modulate Th2 cell cytokine production (18), in this study, Th1 inhibition is present even with active Th2 cell function.

In mice that received cotransfer of Th1+Th2 cells, inhibition of mucus staining was associated with markedly reduced expression of *muc5AC*, the predominant airway mucin gene induced in allergic inflammation (Fig. 1B, 1C). Reduced chitinase activity was associated with a striking reduction in gene expression of *AMCase*, the most abundant chitinase released in the murine respiratory tract (Fig. 1B). When IFN- $\gamma$  effects were eliminated in recipient mice deficient of IFN- $\gamma$ R, mucus and chitinase activity were no longer inhibited (Fig. 1A). Th1 cells, through production of IFN- $\gamma$ , profoundly inhibit the production of mucus and chitinases.

Eosinophils in the airway and lung (data not shown) were both low in C57BL/6 mice after cotransfer of Th1+Th2 cells compared with mice that received Th2 cell transfer alone (Fig. 1A). Eosinophilia was partially restored in *ifngr1*<sup>-/-</sup> mice, indicating that IFN- $\gamma$  and other factors produced by Th1 cells inhibit eosinophils in C57BL/6 mice. Tissue eosinophilia is a complex process that requires eosinophil expansion in the BM, release and recruitment, and in the lung, these effects are mediated in large part by Th2 cytokines and eotaxin-1 (CCL11) (9). Th2 cell transfer and inhaled OVA stimulated increased BM eosinophilopoiesis, and this was blocked in mice that received Th1+Th2 cells (Fig. 1B). Eosinophil chemotactic activity in the BALF was increased in mice that received Th2 cells alone and was inhibited after transfer of Th1+Th2 cells (Fig. 1B). Inhibition of chemotaxis in mice that received Th1+Th2 cells was not explained by reduced eotaxin-1, because eotaxin-1 levels were higher in mice that received Th1+Th2 cells compared with Th2 cells alone (Th2, 12  $\pm$  0.2)



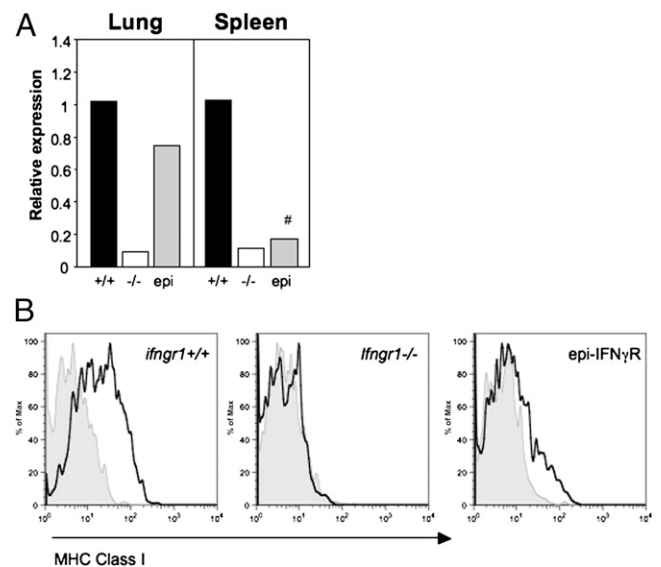
**FIGURE 1.** Th1 cells and IFN- $\gamma$  inhibit Th2-induced airway pathology. WT (C57BL/6) or *ifngr1*<sup>-/-</sup> mice received Th1, Th2, or Th1+Th2 cells and inhaled OVA. **A**, HMI calculating airway mucus staining in lung sections, chitinase activity in BALF and BAL eosinophils ( $n = 5$  mice/group). **B**, qPCR analysis of *muc5AC* and *AMCase* expression in whole lung ( $n = 2$ /group). Eosinophils in the BM and eosinophil migration in response to BALF ( $n = 2-4$ /group). **C**, Mucus staining of lung sections stained with PAS (scale bar, 100  $\mu$ m) ( $n = 5$ /group). Mean levels ( $\pm$  SEM) are shown. These data are each representative of three independent experiments. Chitinase activity in naive mice: WT, 165 U; *ifngr1*<sup>-/-</sup>, 220 U. \* $p < 0.015$ , Th1+Th2 compared with Th2; # $p < 0.0003$ , Th1+Th2 compared with Th1;  $\infty p = 0.039$ , Th1+Th2 compared with Th2.

pg/ml versus Th1+Th2, 1335 [ $\pm$  337] pg/ml;  $p = 0.06$ ). The IFN- $\gamma$ -inducible chemokine inflammatory protein-10 (CXCL10) was increased in the BAL of mice that received Th1+Th2 cells compared with Th2 cells alone (Th2, 202 [ $\pm$  10] pg/ml versus Th1+Th2, 9791 [ $\pm$  1378] pg/ml;  $p = 0.02$ ), and past studies suggest that through binding to CXCR3, CXCL9 and CXCL10 can control eosinophil recruitment (27). When Th1+Th2 cells were transferred cells into mice deficient in CXCR3, followed by inhaled OVA exposure, eosinophils were equivalently reduced in *cxcr3*<sup>+/+</sup> and *cxcr3*<sup>-/-</sup> animals (Supplemental Fig. 2). Therefore, reduced chemoattraction of eosinophils after cotransfer of Th1+Th2 cells is not due to a reduction in CCL11 or inhibitory effects of CXCR3-dependent chemokines. In summary, Th1 cells, by production of IFN- $\gamma$ , reduce Th2-induced mucus and chitinase production and inhibit airway eosinophilia by reducing eosinophil generation and chemotaxis. These data show that IFN- $\gamma$  inhibits three critical pathologic Th2 effects in the respiratory tract by acting at both local and distant sites.

*Generation of mice with IFN- $\gamma$ R expressed only on airway epithelial cells*

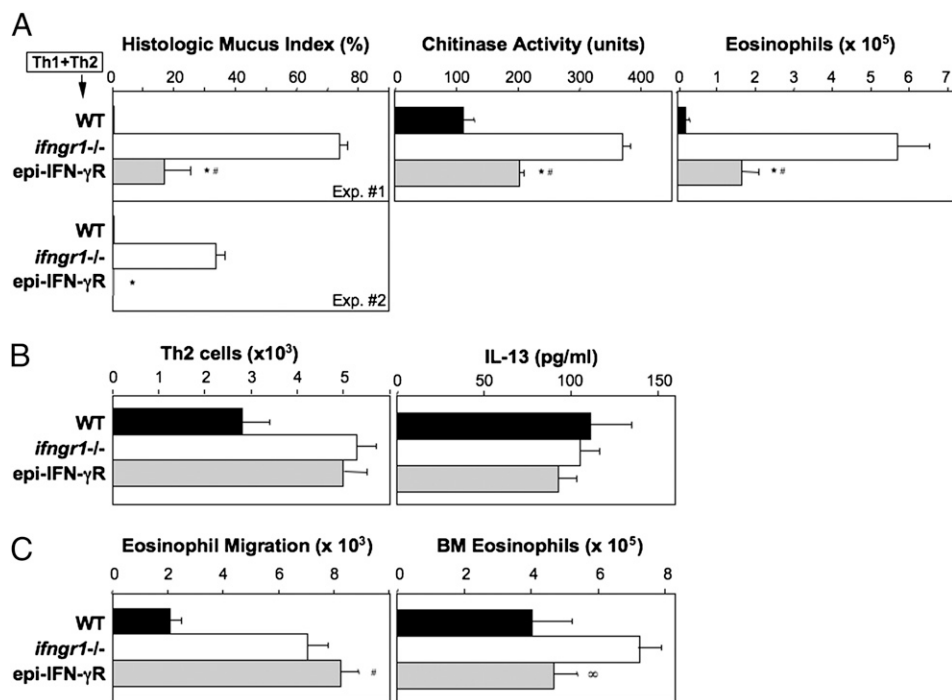
Given the contribution of the airway epithelium to mucus and chitinase production and its importance in immune modulation in the lung, we hypothesized that IFN- $\gamma$ , acting through airway epithelial cells, could provide potent inhibition of Th2-induced effects in the respiratory tract. We generated epi-IFN- $\gamma$ R mice that express IFN- $\gamma$ R only on airway epithelial cells. Transgenic mice were generated by construction of a plasmid containing murine *ifngr1* cDNA under control of a constitutively activated rat CC10 promoter (24). Transgene-positive mice were backcrossed to *ifngr1*<sup>-/-</sup> mice to generate epi-IFN- $\gamma$ R mice. IFN- $\gamma$ R expression was high in the

lungs from *ifngr1*<sup>+/+</sup> and epi-IFN- $\gamma$ R mice and very low in the spleens of both epi-IFN- $\gamma$ R and *ifngr1*<sup>-/-</sup> mice (Fig. 2A), indicating lung-specific expression of IFN- $\gamma$ R in epi-IFN- $\gamma$ R mice.



**FIGURE 2.** Airway epithelial-specific expression of IFN- $\gamma$ R in epi-IFN- $\gamma$ R mice. **A**, qPCR for *ifngr1* expression in whole-lung RNA from naive *ifngr1*<sup>+/+</sup>, *ifngr1*<sup>-/-</sup>, and epi-IFN- $\gamma$ R mice. **B**, MHC class I expression on epithelial cells (gated on CD45<sup>-</sup> and cytokeratin<sup>+</sup> cells) from lungs of *ifngr1*<sup>+/+</sup>, *ifngr1*<sup>-/-</sup>, and epi-IFN- $\gamma$ R mice after 3 d of treatment with inhaled IFN- $\gamma$  or PBS. Black line, IFN- $\gamma$  treated; gray shaded, PBS treated. Data are representative of three independent experiments ( $n = 2-3$ /group). # $p < 0.015$ , epi-IFN- $\gamma$ R compared with *ifngr1*<sup>+/+</sup> mice.

**FIGURE 3.** Th1 cells inhibit Th2-induced airway pathology. WT (C57BL/6), *ifngr1*<sup>-/-</sup>, or epi-IFN- $\gamma$ R mice received Th1+Th2 cells and inhaled OVA. A, HMI (two experiments are shown) and chitinase activity in BALF and BAL eosinophils were assessed ( $n = 5$  mice/group). B, Th2 cell number and IL-13 were measured in BAL. C, Eosinophil migration and BM eosinophils. These data are representative of three independent experiments. Mean levels ( $\pm$  SEM) are shown. Chitinase activity in naive mice: WT, 165 U; *ifngr1*<sup>-/-</sup>, 220 U; epi-IFN- $\gamma$ R, 150 U. \* $p < 0.015$ , epi-IFN- $\gamma$ R compared with *ifngr1*<sup>-/-</sup> mice; # $p < 0.015$ , epi-IFN- $\gamma$ R compared with *ifngr1*<sup>+/+</sup> mice;  $\infty p = 0.02$ , epi-IFN- $\gamma$ R compared with *ifngr1*<sup>-/-</sup> mice.



To show that epi-IFN- $\gamma$ R mice expressed functional IFN- $\gamma$  in the lung, mice were treated with inhaled IFN- $\gamma$  for 3 d, followed by isolation of lung cells and analysis of surface expression of MHC Ags. MHC class I (Fig. 2B) and class II (data not shown) expression were upregulated on the cell surface of epithelial cells isolated from the lungs of *ifngr1*<sup>+/+</sup> mice, whereas in *ifngr1*<sup>-/-</sup> mice, MHC expression was low. In epi-IFN- $\gamma$ R mice, a subpopulation of epithelial cells exhibited upregulation of MHC class I. This subpopulation was consistently 20–22% of lung epithelial cells over multiple experiments. CC10 expression was observed in a comparable percentage of epithelial cells. These data show that epi-IFN- $\gamma$ R mice exhibit restricted IFN- $\gamma$  expression in the lung and restored IFN- $\gamma$ -induced functions in airway epithelial cells.

#### Inhibition of asthma-like features in epi-IFN- $\gamma$ R mice

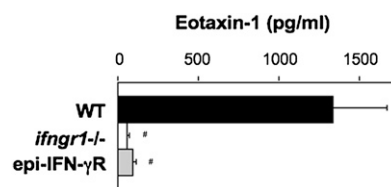
To determine whether IFN- $\gamma$  produced by Th1 cells regulates Th2-induced effects via the airway epithelium, we transferred Th1+Th2 cells into epi-IFN- $\gamma$ R mice, followed by exposure to inhaled OVA. In epi-IFN- $\gamma$ R mice, there was near total inhibition of mucus (Fig. 3A, experiment number 1), similar to our observations in *ifngr1*<sup>+/+</sup> mice. In an experiment in which Th2 induction of mucus was more modest, Th1 cells totally inhibited mucus (Fig. 3A, experiment number 2). Thus, in mice with expression of IFN- $\gamma$ R only on airway epithelial cells, there is complete restoration of the inhibitory actions of IFN- $\gamma$  on mucus. We have previously shown that mucus induction in this model is IL-13 dependent (28); therefore, to ensure that lung Th2 cell infiltration and activation were not different in these strains of mice, we measured Th2 cytokines and Th2 cell numbers in the airways. Comparable levels of IL-13 and Th2 cells were present in BAL from wild-type (WT), *ifngr1*<sup>-/-</sup>, and epi-IFN- $\gamma$ R mice (Fig. 3B), indicating that differential activation and/or recruitment of Th2 cells was not the cause of Th1 inhibition of mucus in epi-IFN- $\gamma$ R mice.

In epi-IFN- $\gamma$ R mice that received Th1+Th2 cells and inhaled OVA, chitinase activity was significantly lower than in mice totally lacking IFN- $\gamma$ R, but complete inhibition was not achieved in any experiment (Fig. 3A).

In epi-IFN- $\gamma$ R mice that received Th1+Th2 cells, BAL eosinophils were reduced almost to the level observed in *ifngr1*<sup>+/+</sup> mice, and this was strikingly lower than observations in *ifngr1*<sup>-/-</sup> mice (Fig. 3B). IFN- $\gamma$ , signaling only through airway epithelial cells, modulates eosinophilia. These studies show that IFN- $\gamma$ , through its actions on the airway epithelium, has potent effects. IFN- $\gamma$  acting through airway epithelial cells totally inhibits airway epithelial mucus production and has partial, but dramatic, inhibitory effects on airway chitinase activity and eosinophils.

#### IFN- $\gamma$ acting on the airway epithelium inhibits eosinophilopoiesis

We next asked whether IFN- $\gamma$ -mediated signals from the airway epithelium inhibit eosinophil development and/or recruitment to the respiratory tract. When mice received Th1+Th2 cells and inhaled OVA, comparable numbers of eosinophils were recovered from the BM of epi-IFN- $\gamma$ R and WT mice, and this was lower than BM eosinophils recovered from *ifngr1*<sup>-/-</sup> mice (Fig. 3C). In contrast, equivalent eosinophil migration was observed in response to BALF from epi-IFN- $\gamma$ R and *ifngr1*<sup>-/-</sup> mice that received Th1+Th2 cell transfer. This suggests that inhibition of airway eosinophils in epi-IFN- $\gamma$ R mice is not due to an IFN- $\gamma$ -induced effect on chemotactic factors but is due to an epithelial-driven effect on the BM that inhibits eosinophilopoiesis. Levels of the Th2 cytokine, IL-5, were comparable in the BAL in the three



**FIGURE 4.** Eotaxin-1 does not correlate with lung and BM eosinophils. Eotaxin-1 in BAL from WT (C57BL/6), *ifngr1*<sup>-/-</sup>, or epi-IFN- $\gamma$ R mice that received Th1+Th2 cells and inhaled OVA. Mean ( $\pm$  SEM) is shown ( $n = 2$ –8/group). Data are representative of three experiments. # $p < 0.00002$ , epi-IFN- $\gamma$ R or *ifngr1*<sup>-/-</sup> compared with WT mice.

groups of mice (data not shown), as was IL-13 (Fig. 3B), again, indicating equivalent pulmonary Th2 cell activation. We also measured eotaxin-1, because, in addition to its chemokine function, it enhances BM eosinophil differentiation (29), and Th2-induced airway epithelial cell production of CCL11 is inhibited by IFN- $\gamma$  (30). Eotaxin-1 was equally low in the BAL from *ifngr1*<sup>-/-</sup> and epi-IFN- $\gamma$ R mice that received Th1+Th2 cells and very high in WT mice (Fig. 4), indicating eotaxin-1 was unlikely to be controlling eosinophil production in these studies. Because only airway epithelial cells can respond to IFN- $\gamma$  in epi-IFN- $\gamma$ R mice, IFN- $\gamma$  appears to stimulate secretion of a factor that controls eosinophil development in the BM.

## Discussion

These studies show that the airway epithelium has an extensive range of effects in regulating pulmonary allergic inflammatory responses. IFN- $\gamma$  acting on the airway epithelium inhibits Th2 cytokine effects in epithelial cells and reduces production of eosinophils. Thus, the airway epithelium, responding to IFN- $\gamma$  from Th1 cells, blocks Th2-induced mucus and chitinase activity locally in the epithelium and remotely in the BM to modulate allergic inflammation. These studies suggest that it may be possible to block some of the pathologic features of asthma using inhaled IFN- $\gamma$ .

Mucus hypersecretion is a major cause of airway obstruction in asthma leading to cough and wheezing and, in exacerbations, is likely a major cause of death (6). Mucus is produced in mucous glands and by goblet cells in the airways. Goblet cells differentiate from Clara or ciliated airway epithelial cells and require epidermal growth factor receptor activation, IL-13, and phosphorylation of Stat6 (7, 31, 32). IFN- $\gamma$  may inhibit mucus production through various effects. Past studies have shown that IFN- $\gamma$  reduces Stat6 phosphorylation in airway epithelial cells (30). Although there is no consensus sequence for Stat6 in the Muc5AC promoter (33), reducing Stat6 phosphorylation may modulate downstream transcription factors, such as SAM pointed domain-containing Ets transcription factor and/or FoxA2, which are essential for goblet cell induction (7, 34, 35). IFN- $\gamma$  also promotes mucous cell apoptosis (36). Therefore, IFN- $\gamma$ , by direct actions on the epithelium, may limit goblet cell development, block mucin gene expression, and/or stimulate loss of mucous cells. Importantly, reduced mucus by any means will have a dramatic impact on airway obstruction.

Chitinases are proinflammatory mediators in the lung. Chitinase activity is driven by Th2 cytokines, specifically IL-13 (10). We have shown that IFN- $\gamma$  inhibits Th2-induced chitinase activity and AMCcase transcription. To our knowledge, these are the first studies to show that chitinases are regulated by IFN- $\gamma$ . Because Th2 cell activation in the lung stimulates chitinase production in airway epithelial cells and macrophages, partial inhibition of chitinase activity may reflect IFN- $\gamma$ -mediated inhibition in airway epithelial cells but not in macrophages (37). Th2-induced chitinase activity is IL-13 and IL-4R $\alpha$ 1 dependent; therefore, it is possible that IFN- $\gamma$  inhibits chitinases through negative effects on Stat6 phosphorylation in a manner similar to the effects on mucus described above. Past studies showed a partial reduction in chitinase activity dramatically reduced inflammation (10). The capacity of IFN- $\gamma$  to reduce chitinases adds to the anti-inflammatory functions of IFN- $\gamma$  in asthma.

Eosinophil recruitment to the respiratory tract in asthma is a complex process coordinated by cytokines that stimulate eosinophil development, release, and recruitment to the respiratory tract (9). The airway epithelium controls cellular inflammation in the respiratory tract principally by release of factors that regulate cell recruitment, such as chemokines. For example, in mice with CC10-driven deletion of the transcription factor FoxA2, there

was increased airway eosinophilia associated with expression of eotaxins (38). Mice with conditional deletion of Stat3 had reduced eosinophilia associated with a reduction in Th2 accumulation and the Th2 chemokine TARC (39). The ability of the airway epithelium to modulate systemic processes in the BM in allergic disease may have been theorized, because the airway produces eotaxin-1 and minute amounts of IL-5 (29, 40), but to our knowledge, this has not been shown in vivo.

Many studies have shown that IFN- $\gamma$  can inhibit eosinophilia induced in allergic disease. These data support the ability of IFN- $\gamma$  to block Th2 cell cytokine production (18) and CD4 T cell recruitment to the lung (19). Although epi-IFN- $\gamma$ R mice exhibited fewer lung and airway eosinophils than *ifngr1*<sup>-/-</sup> mice after Th1+Th2 cell transfer, Th2 cells and Th2 cytokines were equivalent. Chemokines produced by airway epithelial cells, such as eotaxins, influence eosinophil recruitment to the lung, thus making them a prime target of IFN- $\gamma$  effects. Although IFN- $\gamma$  inhibited human eotaxin-3 (30), its effects on eotaxin-1 were variable in vitro, some studies showing IFN- $\gamma$  synergizes with other cytokines to increase its expression (41–44). In this study, in *ifngr1*<sup>+/+</sup> mice, Th2-induced eotaxin-1 was markedly increased by Th1 cells through IFN- $\gamma$  and was associated with low eosinophils. This indicates that IFN- $\gamma$  inhibited eosinophilia independent of eotaxin-1. Furthermore, eosinophil chemotaxis in response to BALF from *ifngr1*<sup>-/-</sup> and epi-IFN- $\gamma$ R was comparable, suggesting that the reduction in eosinophils in epi-IFN- $\gamma$ R mice was not due to differential eosinophil recruitment. BM eosinophils were fewer in epi-IFN- $\gamma$ R mice, indicating reduced development or increased release of eosinophils. Because blood eosinophils were not different among the mice that received Th1+Th2 cells (data not shown), this indicates that IFN- $\gamma$ R activation in the airway epithelium resulted in reduced BM eosinophil production. Eosinophil induction in the BM in allergic disease is dependent on IL-5, which was comparable in BAL in these mice. Eotaxin-1 has also been shown to regulate BM eosinophil development (29), yet there was a lack of association of eotaxin-1 and BM eosinophils. Other possible IFN- $\gamma$ -driven epithelial effects on eosinophil development include reduced production of other proeosinophil cytokines, induction of factors that affect responsiveness to cytokines in the BM, or production of inhibitors of eosinophil development. Importantly, we show that the airway mucosal surface remotely controls eosinophil development to serve its local needs.

These studies show that IFN- $\gamma$  has potent local and systemic effects through its actions on the airway epithelium. Studies of inhaled IFN- $\gamma$  in humans show that the effects are compartmentalized in the respiratory tract (45). Mild asthmatics that received inhaled IFN- $\gamma$  over 3 wk exhibited a reduction in airway eosinophils without evidence of an increase in proinflammatory markers (46). Thus, the local effects of inhaled IFN- $\gamma$  that were observed in asthmatics may represent anti-inflammatory, IFN- $\gamma$ -induced airway epithelial-specific responses, such as those that we report in this paper. These studies support a role for inhaled IFN- $\gamma$  as adjunctive therapy in persistent eosinophilic asthma.

## Disclosures

The authors have no financial conflicts of interest.

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