IFN- γ Blocks Development of an Asthma Phenotype in Rhinovirus-Infected Baby Mice by Inhibiting Type 2 Innate Lymphoid Cells

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Abstract

Early-life wheezing-associated infections with rhinovirus (RV) have been associated with asthma development in children. We have shown that RV infection of 6-day-old mice induces mucous metaplasia and airways hyperresponsiveness, which is dependent on IL-13, IL-25, and type 2 innate lymphoid cells (ILC2s). Infection of immature mice fails to induce lung IFN- γ expression, in contrast to mature 8-week-old mice with a robust IFN- γ response, consistent with the notion that deficient IFN- γ production in immature mice permits RV-induced type 2 immune responses. We therefore examined the effects of intranasal IFN- γ administration on RV-induced ILC2 expansion and IL-13 expression in 6-day-old BALB/c and IL-13 reporter mice. Airway responses were assessed by histology, immunofluorescence microscopy, quantitative polymerase chain reaction, ELISA, and flow cytometry. Lung ILC2s were also treated with IFN- γ *ex vivo*. We found that, compared with

untreated RV-infected immature mice, IFN- γ treatment attenuated RV-induced IL-13 and Muc5ac mRNA expression and mucous metaplasia. IFN- γ also reduced ILC2 expansion and the percentage of IL-13-secreting ILC2s. IFN- γ had no effect on the mRNA or protein expression of IL-25, IL-33, or thymic stromal lymphoprotein. Finally, IFN- γ treatment of sorted ILC2s reduced IL-5, IL-13, IL-17RB, ST2, and GATA-3 mRNA expression. We conclude that, in immature mice, IFN- γ inhibits ILC2 expansion and IL-13 expression *in vivo* and *ex vivo*, thereby attenuating RV-induced mucous metaplasia. These findings demonstrate the antagonistic function of IFN- γ on ILC2 expansion and gene expression, the absence of which may contribute to the development of an asthma-like phenotype after early-life RV infection.

Keywords: asthma; IL-25; neonatal; rhinovirus; type 2 innate lymphoid cells

Recent studies suggest that type 2 innate lymphoid cells (ILC2s) play a direct role in the development of allergic airway responses. The “innate cytokines” IL-25 (or IL-17E) (1–4), IL-33 (1–3, 5, 6), and thymic stromal lymphoprotein (TSLP) (6) each induce activation and production of type 2 cytokines from ILC2s.

Early-life respiratory viral infection, particularly rhinovirus (RV) infection, is a risk factor for asthma development in children (7, 8). To study this, we exposed 6-day-old immature mice to RV1B, a minor group virus that infects mouse cells *in vitro* (9, 10) and *in vivo* (11). We found that neonatal RV infection induced persistent mucous metaplasia and airways

hyperresponsiveness, which was dependent on the type 2 cytokine IL-13 (12). More recently, we showed that RV-induced mucous metaplasia and airways hyperresponsiveness were dependent on airway epithelial cell IL-25 production and expansion of IL-13-producing ILC2s (13). Airway responses were blocked by neutralizing antibody against IL-25.

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Clinical Relevance

Rhinovirus infection of immature mice but not mature mice induces an asthma-like phenotype characterized by increased expression of IL-13 and IL-25, expansion of type 2 innate lymphoid cells, and the absence of a normal IFN- γ response. Restoration of IFN- γ prevents the development of the asthma phenotype, establishing IFN- γ deficiency as a cause of viral-induced airways disease in immature animals. Further characterization of this cellular immune pathway may lead to the identification of molecular and cellular targets for the prevention of asthma.

The newborn immune system is skewed toward type 2 responses. Neonatal T cells and monocytes demonstrate deficient IFN- γ production (14–16), as well as diminished responses to IFN- γ stimulation (17, 18). Accordingly, we found that IFN- γ production was suppressed in RV-infected immature 6-day-old mice in comparison with 21-day-old and mature 8-week-old mice (13). Infants with reduced blood mononuclear cell production of IFN- γ , the principal type 1 cytokine, have been shown to be at a greater risk of recurrent virus-induced wheezing and asthma development (19–22). On this basis, we hypothesized that deficient IFN- γ production in immature mice permits RV-induced type 2 immune responses. To test this, we examined the effects of IFN- γ on RV-induced ILC2 expansion and IL-13 production in immature mice.

Materials and Methods

Generation of RV and Infection of Immature Mice

RV1B (ATCC, Manassas, VA) was partially purified from infected HeLa cell lysates by ultrafiltration using a 100-kD cut-off filter and titered by plaque assay (10, 11). BALB/c and C.129S4(B6)-*Il13^{tm1(YFP/cre)Lky/J}* mice expressing yellow fluorescent protein (YFP) under the control of the IL-13 reporter (2) (Jackson Laboratories, Bar Harbor, ME) were inoculated through the intranasal route under Forane anesthesia with 20 μ l of RV1B (2×10^6 PFU)

or an equal volume of sham HeLa cell lysate. Selected mice were treated with intranasal IFN- γ (10 ng/d) on Days 1, 3, and 5 after RV infection. Experiments were approved by the University of Michigan Institutional Animal Care and Use Committee.

Histology and Immunofluorescence Microscopy

Lungs were perfused through the pulmonary artery with phosphate-buffered

saline containing 5 mM disodium ethylene diamine tetraacetate. Next, lungs were fixed with 4% paraformaldehyde overnight. Five-micrometer-thick paraffin sections were processed for histology or fluorescence microscopy, as described (23). Lung sections were stained with hematoxylin and eosin or Periodic acid-Schiff (PAS) to visualize mucus (Sigma-Aldrich, St Louis, MO). Other lung sections were incubated with Alexa Fluor 488-conjugated mouse

Figure 1. Cytokine expression after rhinovirus (RV) infection. Six-day-old and 8-week-old mice were inoculated with sham or RV, and lung mRNA expression was measured 1 or 4 days later. ($n = 3$ –4 from three different experiments). Data are presented as mean $\pm$ SEM. *Different from sham, one-way analysis of variance. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 2. IFN- γ reduced mucous metaplasia and lung mRNA expression after neonatal RV infection. (A) Periodic acid–Schiff (PAS)–stained lung sections prepared 3 weeks after inoculation of 6-day-old mice with sham + PBS, RV + PBS, or RV + IFN- γ (original magnification $\times 200$). PAS staining was quantified using National Institutes of Health ImageJ software. Staining is represented as the number of cells per millimeter of basement membrane length. A total of 70 airways were analyzed ($n = 5\text{--}7$ mice/group from four different experiments). *Different from sham + PBS; †different from RV + PBS; $P < 0.05$; one-way ANOVA. Scale bar, 100 μm . (B) Lung mRNA expression 3 weeks after inoculation ($n = 4\text{--}5$ from two different experiments). Data are presented as mean $\pm$ SEM. *Different from sham + PBS; †different from RV + PBS; $P < 0.05$; one-way ANOVA. (C) Immunofluorescence staining for Muc5ac (original magnification $\times 100$). Muc5ac staining was quantified using ImageJ software. Staining is presented as the percentage of epithelial basement membrane covered with Muc5ac-positive cells. A total of 34 airways were analyzed ($n = 4$ mice/group from two different experiments). *Different from sham + PBS; †different from RV + PBS; $P < 0.05$; one-way ANOVA.

anti-Muc5ac (clone 45M1; ThermoFisher, Waltham MA), Alexa Fluor 488–conjugated rabbit antimouse IL-25/IL-17E (Millipore, Billerica, MA), or Alexa Fluor 488–conjugated isotype control IgGs.

Flow Cytometric Analysis

Lung cells were stained with Pacific Blue–conjugated antibodies for lineage markers (CD3 ϵ , TCR β , B220/CD45R, Ter-119, Gr-1/Ly-6G/Ly-6C, CD11b, CD11c,

F4/80, and Fc ϵ RI α , from Biolegend, San Diego, CA), anti-CD25-PerCP-Cy5.5 (Biolegend), and anti-CD127-PE-Cy5 (eBioscience, San Diego, CA). Cells were fixed, subjected to flow cytometry, and

Figure 3. Viral copy number in RV-infected and IFN- γ -treated neonatal mice. Six-day-old mice were inoculated with RV + PBS or RV + IFN- γ intranasally. At specified times, lungs were harvested for analysis. Viral copy number was analyzed by quantitative polymerase chain reaction. Shown are individual data from two individual experiments, medians, and interquartile ranges for each time point. There was no significant difference between groups at any time point.

analyzed on an LSR Fortessa (BD Biosciences, San Jose, CA). For analysis of intracellular IL-13, fresh aliquots of isolated cells from lung-minced tissues were stimulated for 5 hours with a cell-stimulation cocktail (40.5 μ M phorbol 12-myristate 13-acetate, 670 μ M ionomycin, 5.3 μ M brefeldin A, and 1 μ M monensin; eBioscience), fixed, permeabilized, and incubated with antimouse IL-13 clone eBio13A (eBioscience). Alexa 488-conjugated anti-GFP (Biolegend) was used to detect the intracellular YFP.

Harvesting of Lung ILC2s

To study lung ILC2s *ex vivo*, lung cells were stained with biotin-conjugated antibodies for lineage markers (CD3 ϵ , TCR β , B220/CD45R, Ter-119, Gr-1/Ly-6G/Ly-6C, CD11b, CD11c, F4/80, and Fc ϵ RI α) and mixed with antibiotin microbeads (Miltenyi Biotech, Auburn, CA). The cell mixture was then subjected to a MACS Separator (Miltenyi Biotech). The flow-through was collected for fluorescence-activated cell sorting. Lineage-negative CD25 and CD127 double-positive ILC2s were sorted at 30,000 cells/ml into 12-well plates and primed with media and IL-2 (50 ng/ml) in the presence or absence of IFN- γ (10 ng/ml) for 24 hours. After priming, IL-25 (20 ng/ml) or IL-33 (20 ng/ml) was added for 24 hours to activate ILC2s.

Real-Time Quantitative Polymerase Chain Reaction

Lung or ILC2 RNA was extracted with Trizol method (Invitrogen, Carlsbad, CA), with the combination of on-column digestion of genomic DNA (Qiagen, Valencia, CA). Complementary DNA was synthesized from 1 μ g of RNA and subjected to quantitative real-time polymerase chain reaction by using specific mRNA primers for IFN- γ , TNF- α , IL-13, IL-5, IL-25, IL-33, TSLP, IL-1 β , IFN- β , Muc5ac, IL17RB, ST2, and GATA-3 (see Table E1 in the online supplement). For each sample, the level of gene expression was normalized to its own glyceraldehyde-3-phosphate dehydrogenase mRNA.

Quantitative one-step real-time polymerase chain reaction for positive-strand viral RNA was conducted using RV-specific primers and probes (forward primer: 5'-GTG AAG AGC CSC RTG TGC T-3'; reverse primer: 5'-GCT SCA GGG TTA AGG TTA GCC-3'; probe: 5'-FAM-TGA GTC CTC CGG CCC CTG AAT G-TAMRA-3') (24). Copy numbers of positive-strand viral RNA were normalized to 18S RNA, which was similarly amplified using gene-specific primers and probes.

Measurement of IL-13, IL-25, IL-33, TSLP, IL-5, and IL-17A Protein Levels

Cytokine levels were measured by ELISA (eBioscience catalog numbers 88-7137-22, 88-7002-22, 88-7333-22, 88-7490-22, 88-7054-22, and 88-7371-22, respectively). ELISA data were analyzed by BioTek Gen5 software (Winooski, VT).

Airway Morphometry

Levels of PAS, Muc5ac, and IL-25 staining in the airway epithelium were quantified by National Institutes of Health ImageJ software (Bethesda, MD). One section from the middle region of the left lung from each mouse was analyzed. PAS staining was represented as the number of PAS⁺ cells per millimeter of basement membrane length. Muc5ac and IL-25 expression was represented as the percentage of IL-25⁺ epithelium compared with the total basement membrane length.

Data Analysis

Data are represented as mean $\pm$ SE. Statistical significance was assessed by unpaired *t* test or one-way analysis of

variance, as appropriate. Group differences were pinpointed by the Newman-Keuls multiple comparison test.

Results

Attenuated Type 1 Response in RV-Infected Neonatal Mice

We infected 6-day-old and 8-week-old mice with RV1B and analyzed cytokine mRNA expression. In contrast to mature mice, RV infection of neonatal mice did not induce increased expression of the type 1 cytokines IFN- γ and TNF- α (Figure 1). On the other hand, the type 2 cytokine genes IL-5 and IL-13 were induced in immature mice but not in mature mice. mRNA expression of the innate cytokines IL-25 and IL-33, each of which has been implicated in ILC2 expansion (1–6), were also increased in RV-infected immature mice, but not in mature mice. These results confirm that early-life RV infection elicits exaggerated type 2 responses while failing to induce a typical type 1 antiviral response. Finally, RV infection increased IL-1 β and IFN- β mRNA expression in both immature and adult mice.

IFN- γ Reduces Mucous Metaplasia in RV-Infected Neonatal Mice

In our previous report, we found that RV infection of neonatal mice induced mucous metaplasia and airways hyperresponsiveness, which was dependent on the type 2 cytokine IL-13 (12, 13). In this study, we hypothesized that IFN- γ counteracts the observed asthma phenotype. PBS or IFN- γ was delivered intranasally 1, 3, and 5 days after RV infection of 6-day-old mice. There was no PAS staining in the noncartilaginous airways of sham-infected, PBS-treated animals (Figure 2A). All RV-infected, PBS-treated mice showed PAS staining of the airway epithelium, indicative of mucous metaplasia, although there was significant individual variability in the response to infection. Finally, IFN- γ treatment of mice after RV infection nearly abolished airway PAS staining. Lung mRNA expression of IL-13 and the mucus-related gene Muc5ac each increased with RV infection but were inhibited with IFN- γ (Figure 2B). We also found that IFN- γ decreased RV-induced mRNA expression of the IL-25 receptor IL-17RB, suggesting

Figure 4. Lung lineage, CD25⁺, CD127⁺ type 2 innate lymphoid cells (ILC2s) in RV-infected BALB/c immature mice. (A) Six-day-old mice were inoculated with sham or RV. PBS or IFN- γ was administered on Days 1, 3, and 5 after infection. Live ILC2s were identified 14 days later and analyzed as a percentage of lineage-negative and total cells (upper two panels) and total ILC2s per lung (lower panel) ($n = 4-5$ from three different experiments). Data are presented as mean $\pm$ SEM. *Different from sham; $\dagger$ different from RV + PBS; $P < 0.05$; one-way ANOVA. (B) Percentages and total of lineage-negative IL-13⁺, CD25⁺, CD127⁺ double-positive (DP) cells ($n = 4-5$ from three different experiments). Data are presented as mean $\pm$ SEM. *Different from sham; $\dagger$ different from RV + PBS; $P < 0.05$; one-way ANOVA. (C) Two weeks after infection, lungs were harvested for measurement of IL-13 by ELISA ($n = 4-5$ from three different experiments). Data are presented as mean $\pm$ SEM. *Different from sham; $\dagger$ different from RV + PBS; $P < 0.05$; one-way ANOVA. FSC, forward scatter; Lin, lineage; SSC, side scatter.

Figure 5. Effect of IFN- γ treatment on lung IL-25, IL-33, and thymic stromal lymphoprotein (TSLP) after RV infection. (A) Six-day-old mice were inoculated with sham, RV + PBS, or RV + IFN- γ . mRNA was measured 7 days after infection ($n = 4-7$ /group from two different experiments). Data are presented as mean $\pm$ SEM. *Different from sham; $P < 0.05$; one-way ANOVA. (B) Two and 7 days after infection, lungs were harvested for measurement of protein levels by ELISA ($n = 3-4$ from two different experiments). *Different from sham + PBS; $P < 0.05$; one-way ANOVA. (C) Seven days after infection, lungs were stained for IL-25 (green) (original magnification $\times 100$). Airway epithelial IL-25 staining was quantified by ImageJ software. A total of 60 airways were analyzed ($n = 3-6$ mice/group from three different experiments). *Different from sham + PBS; $P < 0.05$; one-way ANOVA.

a possible role for IFN- γ in minimizing IL-25-mediated type 2 immune responses. Finally, immunofluorescence staining verified the inhibitory effect of IFN- γ treatment on airway Muc5ac expression (Figure 2C).

To exclude IFN- γ -mediated antiviral responses as a cause of the observed changes, we examined viral load in the presence or absence of IFN- γ . In contrast to PBS-treated mice, there was no significant

effect of IFN- γ on viral copy number 1–3 days after RV infection (Figure 3).

IFN- γ Inhibits Expansion of IL-13-Expressing ILC2s in RV-Infected Immature Mice

RV infection of 6-day-old mice expands the population of ILC2s, a major source of IL-13 after RV infection (13). We questioned whether IFN- γ suppresses the expansion of

ILC2s. We collected lungs 2 weeks after sham or RV infection and analyzed ILC2s using flow cytometry. We gated on small, live lineage-negative cells using a mixture of hematopoietic lineage markers (CD3 ϵ , TCR β , B220, Ter-119, Gr-1, CD11b, CD11c, F4/80, and Fc ϵ RI α) (Figure 4A). After gating on the lineage-negative population, a discrete population of CD25 and CD127 double-positive ILC2s was

Figure 6. Effect of IFN- γ on ILC2 gene expression. Lungs were collected from RV-infected immature mice, and cell suspensions were sorted for Lin-negative CD25⁺ CD127⁺ ILC2s by fluorescence-activated cell sorting. Sorted ILC2s were stimulated with a combination of IL-2, IL-25, IL-33, or IFN- γ . After 2 days of stimulation, cell supernatants were tested for IL-13 protein, and cell pellets were tested for mRNA expression by quantitative polymerase chain reaction. (A) Effect of IFN- γ on IL-25-induced ILC2 mRNA expression of IL-13, IL-5, TNF- α , GATA-3, IL-17RB, and ST2 ($n = 3$ –6 per group from three different experiments). Data are presented as mean $\pm$ SEM. *Different from IL-2 alone or medium; $\dagger$ different from IL-25; $P < 0.05$; one-way ANOVA. (B) Effect of IFN- γ on IL-33-induced ILC2 mRNA expression of IL-13, IL-5, TNF- α , GATA-3, IL-17RB, and ST2 ($n = 3$ –6 per group from two different experiments). Data are presented as mean $\pm$ SEM. *Different from IL-2 alone or medium; $\dagger$ different from IL-33; $P < 0.05$; one-way ANOVA. (C) Effect of IFN- γ on IL-25- and IL-33-induced ILC2 IL-13 and IL-5 production ($n = 3$ –6/group from two different experiments). Data are presented as mean $\pm$ SEM. *Different from IL-2 alone or medium; $\dagger$ different from IL-25 or IL-33; $P < 0.05$; one-way ANOVA.

found. RV infection significantly increased the number of lineage-negative CD25⁺ CD127⁺ cells (Figure 4A). IFN- γ treatment decreased the ILC2 percentage and number.

For analysis of intracellular IL-13, fresh aliquots of lung mince were stimulated for 5 hours with a cell-stimulation cocktail and stained with anti-IL-13. IFN- γ reduced the percentage of IL-13-expressing Lin-negative, CD25-positive cells and IL-13-expressing Lin-negative CD127-positive cells (Figure 4A). In addition, IFN- γ reduced the number of IL-13⁺ CD25⁺ CD127⁺ double-positive cells (Figure 4B). Finally, IFN- γ significantly decreased RV-induced IL-13 production (Figure 4C). Taken together, these results suggest that IFN- γ suppresses RV-induced expansion of IL-13-expressing ILC2s.

We also examined the effect of IFN- γ on IL-13-expressing ILC2s using IL-13 reporter mice expressing YFP under control of the IL-13 promoter to track IL-13 transcription. After RV infection, a discrete population of CD25 and CD127 double-positive ILC2s was found (see Figures E1A and E1B in the online supplement). As in BALB/c mice, expansion of the lung ILC2 population was decreased in IL-13 reporter mice treated with IFN- γ after RV infection. Further analysis showed that RV infection increased the percentage of Lin-negative, CD25⁺ and Lin-negative, CD127⁺ cells expressing YFP (Figures E1C and E1D). Finally, YFP-expressing Lin-negative, CD25⁺ and Lin-negative, CD127⁺ cells were significantly reduced after IFN- γ treatment, suggesting that IFN- γ blocks transcription from the IL-13 promoter in lung ILC2s.

RV-Induced Expression of Epithelial-Derived Innate Cytokines Is Not Inhibited by IFN- γ

We showed previously that epithelial IL-25 is increased in RV-infected immature mice and is required for the development of mucus metaplasia and airways hyperresponsiveness (13). IL-33 and TSLP may also contribute to the development of allergic airway inflammation by inducing the activation and production of type 2 cytokines from ILC2s (1–3, 5, 6). We therefore questioned whether expression of IL-25, IL-33, or TSLP is inhibited by IFN- γ . Consistent with our previous report, IL-25 mRNA and protein expression was increased in RV-infected 6-day-old mice (Figures 5A and 5B). There was no reduction of IL-25 mRNA in the presence of IFN- γ . RV also increased protein but not mRNA levels of

IL-33 and TSLP. There was no effect of IFN- γ on either mRNA or protein expression of IL-33 or TSLP. Lung immunofluorescent staining showed increased IL-25 protein abundance after RV infection, which was unaffected by IFN- γ (Figure 5C). Quantification of IL-25 immunofluorescence by National Institutes of Health ImageJ software showed no significant effect of IFN- γ on airway IL-25 expression. Taken together, these results show that IFN- γ does not block RV-induced mucous metaplasia in immature mice by inhibiting the expression of epithelial-derived innate cytokines IL-25, IL-33, or TSLP.

IFN- γ Inhibits ILC2 Gene Expression

To better assess the effect of IFN- γ on ILC2 maturation and function, we sorted ILC2s and cultured them in the presence or absence of IFN- γ *ex vivo*. Lineage-negative, CD25- and CD127-double-positive ILC2s were sorted from the lungs of RV-infected baby mice. As reported previously (1–3), in combination with IL-2, RV increased ILC2 mRNA expression of IL-13, IL-5, the IL-25 receptor IL-17RB, the IL-33 receptor ST2, and GATA-3 (Figure 6A). Treatment of IFN- γ suppressed ILC2 gene expression. TNF- α mRNA expression was not regulated by IL-25 or IFN- γ treatment. IL-33-induced mRNA expression was also suppressed by IFN- γ (Figure 6B). Finally, IFN- γ abolished IL-25- and IL-33-induced IL-13 protein expression, as well as IL-25-induced IL-5 protein expression (Figure 6C). These results suggest that IFN- γ has an inhibitory effect on ILC2 maturation and function.

Discussion

The immature immune system is qualitatively different from the adult immune system in that it is refractory to type 1 and permissive to type 2 responses. Neonatal T cells and monocytes demonstrate specific deficiencies in IFN- γ production (14–16). Secretion of the type 1 cytokine IL-12 p35 is suppressed in neonatal dendritic cells, thereby inhibiting the ability to elicit T-cell IFN- γ production (25–28). Sp1 transcription factor binding to a positioned nucleosome (nuc-2) within the IL-12(p35) gene promoter is severely impaired in neonatal dendritic cells because of a defect in chromatin remodeling (27). Nuc-2 remodeling and IL-12(p35) gene transcription are restored on the addition of recombinant IFN- γ . Neonatal cells also

show diminished responses to IFN- γ stimulation (17, 18) because of defective STAT-1 phosphorylation. On the other hand, human cord blood T cells show a permissive chromatin architecture at the IL-13 proximal promoter, favoring transcription (29). Murine neonatal CD4⁺ T cells harbor IL-4/IL-13 regulatory elements, which are epigenetically modified to favor type 2 responses (30). We therefore reasoned that immature mice infected with RV would demonstrate a type 2 immune response, in contrast to mature mice, which demonstrate a normal type 1 viral response. Accordingly, we found that IFN- γ production was suppressed in RV-infected immature 6-day-old mice in comparison with 21-day-old and 8-week-old mice (13). RV infection of immature mice also induced persistent mucous metaplasia and airways hyperresponsiveness, which was dependent on airway epithelial cell IL-25 production and expansion of IL-13-producing ILC2s (13). We further hypothesized that deficient IFN- γ production in immature mice permits RV-induced type 2 immune responses. To test this, we examined the effects of IFN- γ on RV-induced ILC2 expansion and IL-13 production in immature mice. In both wild-type and IL-13 reporter mice, IFN- γ blocked RV-induced ILC2 expansion and IL-13 transcription *in vivo*. IFN- γ treatment reduced the gene expression of cultured ILC2s in response to IL-25 or IL-33. These results demonstrate that IFN- γ negatively regulates both ILC2 expansion and gene expression. Furthermore, restoration of IFN- γ prevents the development of the asthma phenotype, establishing IFN- γ deficiency as a cause of viral-induced allergic disease in immature animals.

Our data confirm new reports showing that IFN- γ inhibits ILC2 expansion and gene expression in response to helminth infection (31, 32). In addition, ILC2 function was noted recently to be increased in type 1 IFN receptor-null mice infected with attenuated influenza A virus (33), demonstrating that type 1 IFNs also negatively regulate ILC2s. Type 1 IFN-mediated inhibition of ILC2 proliferation and cytokine release occurred independently of STAT4, T-bet, iNOS, and STAT6 but required STAT1, STAT2, and IRF9. Our study extends the latter report by showing that a physiologic state of IFN deficiency (i.e., immaturity) also permits virus-induced ILC2-mediated type 2 immunopathology.

Because RV-induced airway responses in 6-day-old mice require virus replication (13), it is possible that IFN- γ attenuates ILC2 responses by inhibiting viral replication. Inhibition of viral replication by IFN- γ is required for disease resistance to encephalomyocarditis virus (34) and Theiler's murine encephalomyelitis virus (35). However, IFN- γ administration had no effect on rhinoviral copy number, suggesting that the antiviral function of IFN- γ did not account for the attenuated ILC2 response. Similarly, airway epithelial cell-derived IL-25 is required for IL-13-dependent mucous metaplasia and airways hyperresponsiveness in RV-infected immature mice (13) and for respiratory syncytial virus-induced Th2 responses in natural killer cell-deficient mice (36), suggesting the possibility that IFN- γ attenuates type 2 responses by blocking IL-25. However, RV-induced lung IL-25 mRNA and protein expression was not reduced in the presence of IFN- γ . On the contrary, we found, we believe for the first time, that IFN- γ blocks ILC2 mRNA expression of the IL-25 receptor IL-17RB, consistent with the notion that IFN- γ blocks the sensitivity of ILC2s to IL-25 secretion. Finally, there was no effect of IFN- γ on the mRNA or protein expression of IL-33 and TSLP, each of which may also regulate the production of type 2 cytokines from ILC2s (1–3, 5, 6). Together, these data show that IFN- γ does

not block the development of the mucous metaplasia phenotype by inhibiting the expression of epithelial-derived innate cytokines.

A shift in the Th1/Th2 cytokine balance toward a type 1 profile may contribute to protection against asthma and allergy. Peripheral blood monocytes from children and adults with asthma produce lower levels of IFN- γ in response to allergen when compared with cells from control individuals (37, 38). IFN- γ levels are increased in allergic patients after immunotherapy (39, 40). Infants with reduced blood mononuclear cell production of IFN- γ , the principal type 1 cytokine, have been shown to be at a greater risk of recurrent virus-induced wheezing and asthma development (19–22). IFN- γ production by stimulated cord blood mononuclear cells is a negative predictor of allergic disorders including asthma (19). Lower blood mononuclear cell IFN- γ production at the time of bronchiolitis has been related to the development of asthma (20). Impaired IFN- γ production at 3 months significantly increases the risk of developing recurrent wheezing in the first year of life (21). Newborns with measurable peripheral blood monocyte IFN- γ responses to respiratory viral infection are less likely to develop recurrent virus-induced wheezing than are those without such responses (22). It is therefore conceivable that, in early life, deficient

IFN- γ production after respiratory viral infection may lead to an aberrant type 2 response favorable to asthma development, particularly in genetically susceptible individuals, when maintained by appropriate stimuli.

Unlike Th2 cells, ILC2s do not express acquired antigen receptors or undergo clonal selection and expansion when stimulated. Still, mature ILC2s express the characteristic surface receptors and effector molecules of differentiated T-cell subsets (41, 42). Like Th2 cells, both human and mouse ILC2s express high levels of GATA-3, and GATA-3 is essential for ILC2 development and Th2 cell cytokine production (43–46). Our *ex vivo* study showed reduced GATA-3 mRNA expression in IFN- γ -treated ILC2s, suggesting a novel mechanism by which IFN- γ may inhibit ILC2 proliferation and cytokine release.

Conclusions

We conclude that IFN- γ treatment inhibits the development of an asthma phenotype in RV-infected immature mice by suppressing the expansion of IL-13-expressing ILC2s. Further characterization of this cellular immune pathway may lead to the identification of molecular and cellular targets for the prevention of asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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