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NK Cell Deficiency Predisposes to Viral-Induced Th2-Type Allergic Inflammation via Epithelial-Derived IL-25

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Severe respiratory syncytial virus (RSV) infection has long been associated with an increased risk for the development of childhood asthma and exacerbations of this disorder. Despite much research into the induction of Th2 responses by allergens and helminths, the factors associated with viral infection that predispose to Th2-regulated asthma remain unknown. Recently, clinical studies have shown reduced numbers of NK cells in infants suffering from a severe RSV infection. Here we demonstrate that NK cell deficiency during primary RSV infection of BALB/c mice results in the suppression of IFN-γ production and the development of an RSV-specific Th2 response and subsequent allergic lung disease. The outgrowth of the Th2 responses was dependent on airway epithelial cell-derived IL-25, which induced the upregulation of the notch ligand Jagged1 on dendritic cells. This study identifies a novel pathway underlying viral-driven Th2 responses that may have functional relevance to viral-associated asthma. The Journal of Immunology, 2010, 185: 4681–4690.

Respiratory syncytial virus (RSV) is a single-stranded, negative-sense RNA virus from the Paramyxoviridae family (1). RSV is the primary cause of hospitalization in the first year of life for children worldwide (1). It has long been recognized that infants suffering from a severe RSV infection, leading to bronchiolitis, are at significantly greater risk for the development of asthma (a CD4 lymphocyte-dominated immune condition) and wheezing in later childhood (1–7). A long-term prospective study from Simoes et al. (8) attempted to delineate further whether this was a causal association by examining respiratory outcomes after passive immunization against RSV using palivizumab. This study revealed that by preventing hospitalization due to severe RSV infection, the risk of recurrent wheezing in later childhood was reduced by 50%. Similarly, treatment with polyclonal immune globulins revealed that by preventing hospitalization due to severe RSV infection, the risk of recurrent wheezing in later childhood was reduced by 50%. Similarly, treatment with polyclonal immune globulins had also been demonstrated to reduce the incidence of childhood asthma 7–10 y later (9). These studies and others suggest that severe RSV infection is not simply a marker of asthma predisposition but instead provide support for a causal relationship between early-life viral exposure and childhood asthma.

An emerging trend born from multiple clinical studies of severely RSV-infected infants is a failure to generate a robust NK cell response (10–13). Furthermore, the magnitude of the deficiency increases with worsening severity of disease indicating an inverse relationship (10, 13). For instance, although the numbers of NK cells were reduced in patients admitted to hospital with RSV bronchiolitis, a subpopulation of infants who were ventilated exhibited an even greater (3-fold) reduction in NK cells, and fatal cases displayed a near absence of NK cells in lung autopsy specimens. Thus, deficiency in NK cell numbers is critically linked to severity of RSV-induced disease.

NK cells were originally thought to solely function as early innate inflammatory cells for host defense against pathogens and malignancy. It is now recognized that these cells play a crucial role in the priming of adaptive immune responses against a variety of viral infections (14). Indeed, the recruitment and activation of IFN-γ-producing NK cells to the site of inflammation plays a critical role in the subsequent development of an effector CD4 Th1 and CTL response (15). This may occur indirectly through NK cell licensing of dendritic cells (DCs). During this bidirectional cross-talk, IFN-γ released by NK cells activates DCs to produce IL-12, which in turn feeds back on the NK cell to further amplify IFN-γ secretion (15, 16). Of note, defective NK cell function is strongly linked with the development of Th2-dominated immune responses in atopic eczema (17–19).

The absence of inflammatory signals in the lung microenvironment has been proposed to default naive T cells toward a Th2 phenotype (20); however, recent reports indicate that specific Th2-inducing mechanisms also exist. Thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 have all been identified as innate immune mediators that promote Th2 differentiation during sensitization to allergens and/or helminthic parasites (21). Although both macrophages and granulocytes secrete TSLP, IL-25, and IL-33, airway epithelial cells are also a rich source of these cytokines supporting the paradigm that the activation of stromal cells influences the development of Th2 immunity (22–24). These cytokines have the ability to induce Th2 cell differentiation by acting either directly on naive T cells or indirectly through modulation of other cell types (22, 23). Although these factors contribute to the generation of Th2
immune responses during exposure to allergens, it remains unknown how viral infections (such as RSV) may induce Th2 rather than protective Th1 responses. Collectively, clinical observations suggest that impaired or deficient NC cell activity may play a role in the manifestation of severe RSV-induced bronchiolitis, a disease often associated with the development of childhood asthma. Data are also emerging suggesting that epithelial cell-derived cytokines (TSLP, IL-25, and IL-33) may promote Th2 immunity at mucosal surfaces. In this study, we examine the role of NC cells in the regulation of RSV infection and in particular how a viral infection may influence the Th2 immune compartment. By employing a mouse model of RSV infection and NC cell depletion, we demonstrate a critical role for this cell in protection against the generation of virus-specific and bystander allergen-specific Th2 responses. Airway epithelial cell-derived IL-25 was significantly upregulated due to the reduced levels of IFN-γ in the absence of NC cells in infected mice, demonstating a unique inverse relationship between these factors. IL-25 was critical to the development of Th2 cells and inflammation in response to RSV, at least in part, by the upregulation of the notch ligand Jagged1 on lymph node DCs. IL-25 activated a novel Th2-inducing pathway during respiratory viral infection.

Materials and Methods

Mice and infections

Male BALB/c mice (8–10 wk-old) were used in all studies. Mice were administered virus via the intranasal route under light isoflurane-induced anesthesia. RSV (long strain, type A) was obtained from the American Type Culture Collection and was propagated in monolayers of HEp-2 cells for 4 d. Supernatant and cells were collected with 50 mM HEPES and 100 mM MgSO4 and centrifuged to produce clarified supernatant. The resulting volume was layered onto a two-step sucrose gradient and ultracentrifuged at 100,000 × g for 1 h at 4˚C. RSV titer was determined by plaque assay. Mice were administered 5 × 106 PFU RSV in 50 μl PBS on day 0. Influenza strain A/PR/8/34 was propagated and purified as previously described (25). Mice were administered 1000 PFU influenza in 50 μl PBS on day 0. All studies were approved and conducted in accordance with guidelines set out by the University of Newcastle Animal Care and Ethics Committee.

Mouse treatment regimens

Mice were administered intraperitoneal injections of anti-ASIALO GM1 (Wako, Los Angeles, CA) on days −1, 1, and 4 to deplete NC cells, or purified Ab clone YTS 169.4 on days −3, −1, 2, and 5 to deplete CD8 T cells, or were treated with isotype-matched controls. Treatments were confirmed by flow cytometry to deplete 95% NC cells and 98% CD8 T cells, respectively. Mice that were depleted of NC cells were also treated in separate experiments with an IL-25 neutralizing Ab clone 35B ( kindly provided by Chen Dong, MD Anderson Cancer Center, Houston, TX) or rat IgG isotype control (Sigma-Aldrich, St. Louis, MO) (both 250 μg in 200 μl PBS) by intraperitoneal injection on days −1, 1, and 3 or were treated with recombinant mouse IFN-γ (BD Biosciences, San Jose, CA) (1 μg in 30 μl PBS) by intranasal instillation on days 1, 2, and 3. To deplete basophils, mice were treated with anti-FcεRI (clone MAR-1) or an isotype control as previously described (26). To determine the impact on an inhaled Ag model, mice were exposed to OVA protein via the intranasal route without adjuvant. Mice were given 100 μg OVA on days 3, 5, and 7 after 2 wk were challenged with 25 μg OVA on days 21, 22, 25, 26, and sacrificed 24 h later.

Lymph node stimulation assays

Lymph node cells were prepared and placed in culture at 2 × 106 cells/well/200 μl growth medium (RPMI 1640, 5 × 10−5 M 2-mercaptoethanol, 10% heat-inactivated FCS, 2 mM l-glutamine, 20 mM HEPES, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM sodium pyruvate). Cells were treated with either 50 μg/ml anti-Jagged1 (Sigma-Aldrich) or a goat IgG isotype control or were left untreated and cultured for 3 d. For the OVA model, lymph node cells were stimulated with 200 μg/ml OVA protein or cultured without stimulation for 4 days.

Purified CD4 T cell stimulation assay

NAIVE T cell-depleted splenocytes were inactivated with mitomycin C treatment according to the manufacturer’s instructions (Sigma-Aldrich). Splenocyte feeder cells were plated at 5 × 105 cells/well/100 μl growth medium and exposed to UV-inactivated RSV (multiplicity of infection = 0.5) or unstimulated. Lymph node cells were isolated from treated mice, and CD4 T cells were purified by negative selection using the CD4 T Lymphocyte Enrichment kit (BD Biosciences) and plated out at 2 × 105 cells/well/100 μl growth medium together with the feeder cells for 3 d culture.

Histology

Lung tissue was fixed in 10% formalin and paraffin-embedded for sectioning. Stains included chromotrope 2R and hematoxylin to enumerate eosinophils or periodic acid-Schiff to enumerate mucus-secreting cells. Cells were counted around major airways for a minimum of 10 fields at ×10 magnification.

Real-time quantitative polymerase chain reaction

Lungs and lymph nodes were excised and frozen in RNAlater solution (Ambion, Austin, TX) at −80˚C. Total RNA was isolated using TRIReagent solution (Ambion) according to the manufacturer’s instructions. Reverse transcription and quantitative PCR were conducted with Superscript III and Express SYBR Green with ROX (Invitrogen, Victoria, Australia) according to the manufacturer’s instructions. Intron spanning primer sequences were designed for the following: IL-4 (5′-TTGGAGGATATCCGATTCG-3′ and 5′-TCAGACAGTGATTCTCCCCATGTG-3′), IL-12p35 (5′-GGTAAACACAGATGACATGGT-3′ and 5′-GGTTAGACGAGACACAGTACGTG-3′), IFN-γ (5′-TCTTGAAGAGAGACATACGGGCTA-3′ and 5′-GAATCGAGGACACCACTCCCTTTCC-3′), Cox-1 (5′-CCCAAGACACACTGTGAAGAAGCTACAAAC-3′ and 5′-TGTGCGCACAATGGCTGGTCT-3′), Cox-2 (5′-TACCAGCTCCGCGCTGTGTCATCTCC-3′ and 5′-TAAACCTCGGTGCTATTCCGACG-3′), hypoxanthine phosphoribosyltransferase (HRPT) (5′-AGGCCAGATGTGTTGATTTGGA-3′ and 5′-CAACTTGCGCTC-ATCTAGGCTTT-3′), and RSV strain N gene (5′-CATCCAGCATAAACCACATCCA-3′ and 5′-TTCCTGACATATAATTAGGCTATCA-3′). Real-time PCR was performed using the Realplex Mastercycler ep (Eppendorf, Hamburg, Germany) using the following cycling conditions: 1 cycle at 50˚C, 2 min; 1 cycle at 95˚C, 2 min; 40 cycles at 95˚C, 15 s; 40 cycles at 60˚C, 1 min; plus standard melting curve. Relative gene expression was determined using an HRPT housekeeping gene, whereas quantification of RSV viral titer was determined using plasmid copy number standards for RSV N gene and HRPT.

Immunohistochemistry

Paraffin-embedded sections were cut, rehydrated, and exposed to Ag retrieval in sodium citrate buffer. Sections were incubated in 0.3% Triton X-100/PBS for 10 min to permeabilize the cells before blocking nonspecific binding with 5% rabbit Igs (Sigma-Aldrich) for 1 h. Sections were washed in 0.3% Triton X-100/PBS and incubated with 60 μg/ml rat anti-mouse IL-25 (clone 35B) or a rat IgG isotype control (Sigma-Aldrich) overnight at 4˚C. Sections were washed and incubated with a 1/100 dilution rabbit anti-rat IgG-biotin (Dako, Glostrup, Denmark) for 1 h and washed and incubated with a 1/100 dilution streptavidin-alkaline phosphatase (Amer sham Biosciences, New South Wales, Australia) for 30 min. Washes in TBS were followed by color development using the Fast Red TR/Naphthol system (Sigma-Aldrich) with levamisole endogenous alkaline phosphatase inhibitor (Dako) and hematoxylin counterstain. Photographs were captured at room temperature at ×40 magnification using an Olympus microscope (model BX51), digital camera (Olympus DP70), and DP software (Olympus, Center Valley, PA).

Flow cytometry/intracellular cytokine staining

Mediastinal lymph node cells or lung cells were isolated and incubated with anti-mouse CD16/32 Fc receptor blocker at 20 μg/ml/106 cells for 15 min on ice. Cells were incubated with the relevant Ab on ice for 20 min in the dark. Abs included anti-mouse MHC class II-allophycocyanin, FcεRI-biotin, CD3-PECy7, e-kl-PECy7 (all from eBioscience, San Diego, CA), D55-PE, DC13-PE, Gr-1-allophycocyanin, CCR3-allophycocyanin, IL-4-PECy7 (all from BD Biosciences), delta-like ligand 4 (dlh)-biotin (R&D Systems, Minneapolis, MN), and purified rabbit anti-mouse Jagged1 (Santa Cruz Biotechnology, Santa Cruz, CA). All isotype control Abs were obtained.
from BD Biosciences. For biotinylated Abs, a streptavidin-conjugated PerCP (BD Biosciences) was added and for Jagged1 a goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen) was added at 4°C for 30 min. Intracellular staining was performed by incubating lymph node cells for 4 h unstimulated in growth media containing brefeldin A (Sigma-Aldrich) at 5 μg/ml. Cells were first stained for DX5, FcεRI, c-kit, CD3, CCR3, Gr-1, fixed, permeabilized, and then stained for IL-4 according to standard intracellular staining procedures before being fixed. Cells were analyzed on a FACS-Canto (BD Biosciences). DCs in the draining lymph nodes were gated based on their expression of CD11c⁺MHCclassII⁺

**Bronchoalveolar lavage analysis**

A cannula was placed into the trachea and bronchoalveolar lavage (BAL) conducted using two 0.7-ml injections of HBSS, cells were spun down, and RBCs were lysed. Cells were then spun onto slides using cytopsin centrifugation and stained with the May–Gru¨nwald and Giemsa stains. A minimum of 400 total cells were counted to determine differential WBC percentages.

**ELISA**

Serum concentrations of total IgE were determined using reagents from BD Biosciences. OVA-specific IgG1 and IgG2a concentrations in the serum and BAL fluid were determined by coating plates with 20 μg/ml OVA protein and using Abs from Southern Biotech (Birmingham, AL). Cell culture supernatants were analyzed for the cytokines IL-4, IL-5, and IFN-γ using Abs from BD Biosciences and IL-13 using Abs from R&D Systems. All standards were obtained from BD Biosciences.

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism version 4.0 software (GraphPad, San Diego, CA) with the Mann-Whitney U test and one-way ANOVA tests. A p value <0.05 was considered statistically significant.

**Results**

**NK cells protect against viral-mediated Th2 allergic airways inflammation**

To investigate the role of NK cells in regulating RSV infection, we compared immune and morphological responses between wild-type (WT) isotype control-treated (WT control) mice and NK cell–depleted mice. The influx of NK cells into the lungs peaked 4 d after inoculation with live RSV, and NK cell numbers were reduced by 95% in the lung (80% in the mediastinal lymph nodes).

**FIGURE 1.** NK cells protect against viral-mediated Th2 responses. Mice were treated with anti-ASIALO GM1 (NK-depleted), YTS169.4 (CD8-depleted), or isotype control (WT control) Abs, as described in Materials and Methods, and mice were inoculated with RSV on day 0. A, At day 4, relative mRNA expression of IFN-γ was measured in the lungs by quantitative PCR and normalized to Hprt. Data are expressed as the fold change over naive mice and represent the mean ± SEM. ***p < 0.01 compared with WT control mice. B, Relative mRNA expression of IL-4, IL-13, and eosinophil-1 and eosinophil-2 were measured in the lungs on day 9. Data represent the mean ± SEM. ***p < 0.001; **p < 0.01; *p < 0.05 compared with WT control mice. C, Total IgE was determined in the serum of mice on day 9. Results represent the mean ± SEM. ***p < 0.001 compared with WT control mice. D, Eosinophils were enumerated in the airway parenchyma per 100 μm basement membrane (BM) on day 9. Data represent the mean ± SEM. *p < 0.05; **p < 0.01 compared with WT control mice. E, The percentage of epithelial cells in the airways staining positive for mucus was enumerated on day 9. Data represent the mean ± SEM. ***p < 0.001 compared with WT control mice. F, At 9 d, feeder splenocytes were pulsed with UV-inactivated RSV and plated out with lymph node CD4 T cells from each mouse. Supernatants were analyzed for IL-4, IL-5, IL-13, and IFN-γ. Data represent the mean ± SEM. *p < 0.05 compared with WT control mice. Results represent two independent experiments with n ≥ 5 mice per group. ND, not detected.
confirming NK cell Ab-mediated depletion (Supplemental Fig. 1A, 1B). Inoculation of NK cell-depleted mice with RSV resulted in significantly reduced levels (5-fold reduction) of IFN-γ mRNA expression in the lungs compared with that in WT control and CD8 T cell-depleted mice at 4 d postinfection (dpi) (Fig. 1A). As determined by intracellular cytokine staining, NK cells directly produce IFN-γ protein and furthermore are the major source of this cytokine in the lungs at 4 d after RSV infection (Supplemental Fig. 2A, 2B). Depletion of NK cells led to increased mRNA levels of the Th2-type cytokines (IL-4 and IL-13) and eosinophil-active chemokines (eotaxin-1 and eotaxin-2) in the lungs at 9 dpi (Fig. 1B).

Furthermore, other classical markers of Th2-mediated allergic inflammation were significantly elevated in the absence of NK cells, as NK-depleted mice had increased serum IgE and increased numbers of tissue eosinophils and mucus-secreting cells (Fig. 1C–E). The increased mucous cell metaplasia in NK-depleted mice also coincided with the appearance of mucus plugging in the airways as is depicted visually in the micrographs of Supplemental Fig. 3. This increased Th2 pathology was reflected in a delayed viral clearance in the later stages of infection (Supplemental Fig. 4). A trend to greater numbers of mast cells was also observed (Supplemental Fig. 5). To demonstrate the specificity of NK cell depletion for the induction of the Th2 immune phenotype, mice were also depleted of IFN-γ-producing CD8 T cells. Depletion of this T cell subset did not promote Th2 responses; however, a small increase in eosinophils was observed (Fig. 1B–E). Thus, the reduction in IFN-γ and the development of an RSV-driven Th2 phenotype was induced specifically through the absence of NK cells and independently of CD8 T cells. Importantly, to confirm that the cellular source of the Th2 cytokines was the differentiation of viral-specific Th2 lymphocytes, CD4 T cells were purified from the mediastinal lymph nodes of mice (9 dpi) and stimulated with feeder splenocytes pulsed with UV-irradiated RSV. This approach eliminated any interference from other potentially virus-activated cells within the lymph nodes. RSV-specific CD4 T cell-derived IL-4, IL-5, and IL-13 were all significantly elevated in the absence of NK cells compared with that in WT controls but not in the absence of CD8 T cells, whereas levels of RSV-specific IFN-γ production were reduced (Fig. 1F). Thus, viral-specific Th2 cells develop in the absence of NK cells.

**Viral-specific Th2 responses persist into long-term memory**

Although NK cells were depleted during a primary infection, they returned to normal levels by 16 dpi (data not shown). In the previously NK cell-depleted mice, a secondary inoculation in the presence of NK cells 42 d after primary RSV exposure induced CD4 T cells to secrete increased levels of the Th2 effector cytokines IL-4 and IL-13 compared with that in WT control mice (Fig. 2A). Furthermore, both the numbers of mucus-secreting cells and tissue eosinophils in the lung were significantly elevated above controls (Fig. 2B, 2C). The morphological changes appear milder than at 9 dpi, which may reflect the presence of NK cells during the secondary infection, but may also reflect the impact of protective immunity to RSV elicited by a strong secondary neutralizing Ab response. Nevertheless, these data demonstrate that the Th2 cells generated by a primary infection in the absence of NK cells can persist over the long term and can be reactivated by viral Ags associated with airway changes characteristic of allergic inflammation.

**NK cell deficiency during RSV infection induces allergic airways inflammation in response to an innocuous bystander Ag**

To examine concurrent exposure to both RSV and airborne allergens (to more closely replicate the situation in humans), we next evaluated the role of NK cells in the modulation of viral infection concomitantly with exposure of the airways to an innocuous Ag. Direct exposure of the airways to intranasal OVA without adjuvant is known to result in immunological tolerance to this protein (27). To determine if the Th2 response generated by RSV infection in the absence of NK cells affected immunological tolerance to inhaled bystander Ag, the WT control or NK cell-depleted mice were infected with RSV while being simultaneously exposed to inhaled OVA (Fig. 3A). In vitro restimulation of lymph node cells from mice that had been exposed to virus and OVA in the absence of NK cells (NK-depleted RSV OVA) induced substantial increases in IL-4, IL-5, and IL-13 production compared with that in the WT control mice (WT RSV OVA) (Fig. 3B). Both the naive and the OVA-alone mice had low levels of all cytokines measured. Hallmark features of allergic inflammation were also observed in NK-depleted RSV OVA mice. The numbers of epithelial cells secreting mucus and eosinophils in the BAL fluid and lung tissue were significantly increased compared with that in WT RSV OVA mice (Fig. 3C–E). Furthermore, the humoral response generated in NK-depleted RSV OVA mice was strongly biased toward Th2, as evidenced by increased serum and BAL fluid concentrations of OVA-specific IgG1 in contrast with decreased levels of OVA-specific IgG2a (Fig. 3F). Notably, mice that did not receive an RSV infection but were nevertheless depleted of NK cells (NK-depleted OVA) produced significantly lower OVA-specific IL-4 and IL-13; however, these mice produced significantly higher levels of IL-5 compared with that in mice infected with RSV. This higher IL-5 secretion was associated with elevated eosinophil numbers in the BAL fluid. This result also indicates a direct role for NK cells in suppressing pulmonary Th2 responses and maintaining tolerance to inhaled Ags. However, the strongest Th2 response (range of cytokines generated, development of a humoral response, and degree of lung inflammation) occurred in the absence of NK cells during exposure to RSV. Thus, NK cells play an
The RSV-induced Th2 immune response is initiated early in the lymph nodes and reversed by IFN-γ

Cytokine expression in the lymph nodes at 4 dpi was examined to determine whether alteration in Th polarization signals occurred during the early phase of T cell differentiation. Indeed, in NK-depleted mice, significant alterations in the mRNA expression of IL-4 (3-fold increased) and of IL-12 and IFN-γ (both 3-fold decreased) were observed in the lymph nodes compared with that in WT control mice (Fig. 4A). Similarly, when these lymph nodes were cultured for 3 d, there was also an increase in the protein level of IL-13 (likewise for IL-4 and IL-5; Supplemental Fig. 6), coupled with a decrease in IFN-γ (Fig. 4B). The skewing of these responses toward Th2 was not due to higher RSV load, as there was no detectable difference in viral titer between WT control and NK-depleted mice during T cell differentiation at 4 dpi (Supplemental Fig. 7). To determine the impact of a reduced level of IFN-γ in the lungs due to the absence of NK cells, NK-depleted mice were treated with recombinant IFN-γ by repeated intranasal instillation. By using this treatment, the cytokine pattern induced by NK cell depletion was significantly reversed to reflect that of WT controls and a Th1 response (Fig. 4A, 4B). These data demonstrate a critical role for the IFN-γ signal from NK cells in the regulation of viral-induced Th2 immune responses. As IL-4 was elevated in the lymph nodes at 4 dpi, and given recent reports of basophils as a major source of IL-4 contributing to the induction of Th2 responses to allergens in the lymph nodes, we determined the influence of this cell type on the development of RSV-driven Th2 pathology. FcReI5CD49b+c-kit−CCR3−Gr-1−CD3− basophils were observed within the lymph nodes of RSV-infected mice at similar percentages as that of influenza-infected and naive control mice (Supplemental Fig. 8). After intracellular staining, approximately 50% of the basophils isolated from RSV-infected mice were found to secrete IL-4 (Fig. 4C). By contrast, those cells from naive or influenza-infected mice did not secrete IL-4 at all, suggesting this to be a unique effect of RSV. However, basophils were recruited to the lymph nodes independently of NK cells, as there was no significant difference between WT control versus NK-depleted mice. Furthermore, depletion of these cells (using MAR-1 mAb) failed to alter the generation of the Th2 phenotype (Fig. 4D). Despite basophil depletion leading to an increase in RSV-specific CD4 T cell IFN-γ, there was no significant effect on the Th2 responses (no significant change in IL-4, IL-5, or IL-13 expression). Therefore, although these IL-4–secreting basophils may make some contribution to the generation of Th2 responses under some conditions, they are not critical to the generation of the RSV-specific Th2 responses.

IL-25 expression is induced in the respiratory epithelium by RSV and sustained in the absence of NK cells and IFN-γ

We next determined the mechanism whereby NK cell/IFN-γ deficiency resulted in the generation of Th2 responses to RSV to answer the question whether there was a Th2 default in the lung in the absence of these factors or whether the NK cell/IFN-γ suppresses a Th2-inducing mechanism. Given the emerging role of the cytokines TSLP, IL-25, and IL-33 in the initiation of Th2 immunity, we measured the expression of these cytokines in the lung compartment at 2 and 4 dpi. Only the levels of the IL-17 family member IL-25 (IL-17E) were significantly increased (~5-fold) in the absence of NK cells, and this correlated with significantly reduced IFN-γ levels in the lung (~5-fold) at this time (Fig. 5A). By contrast, the expression level of the other cytokines (TSLP,
IL-33, and IL-4) involved in polarization of Th2 cells was not altered in the lung (data not shown). When the airways of NK-depleted mice were repeatedly treated with recombinant IFN-γ, levels of IL-25 were significantly reduced both at 2 and 4 dpi (Fig. 5B). The expression pattern of IL-25 and IFN-γ identified a unique inverse relationship, wherein IFN-γ levels remained at baseline whereas IL-25 expression increased at 2 dpi independently of NK cells. When NK cells infiltrate into the lungs at 4 dpi, a disparate IFN-γ response emerges between the RSV-infected WT control and NK cell-depleted mice. As the expression of IFN-γ increases, this suppresses the expression of IL-25. Conversely, in the absence of NK cell influx and IFN-γ production, IL-25 expression is not suppressed and continues to increase in lung tissue. By using immunohistochemistry, we identified that the IL-25 signal was localized to the respiratory epithelium, the same site known to be infected by RSV (Fig. 5C). These immunohistochemistry results also demonstrated that at 4 dpi, WT control mice displayed negligible levels of IL-25, which was comparable with that of the naive mice and also the NK-depleted RSV sections stained with the isotype control Ab, thus confirming the mRNA expression data. Thus, only in the absence of NK cells did RSV maintain a high level of IL-25 expression (4 dpi) in the respiratory epithelium.

**RSV-induced Th2 responses are IL-25 dependent**

To identify a role for IL-25 in the induction of the Th2 response in RSV-infected NK-depleted mice, this cytokine was blocked in vivo. NK-depleted mice were treated with anti–IL-25 mAb or an isotype control. As only a temporary blockade was sought for a specific period, gene-deficient mice were not considered a preferable option. Lymph node CD4 T cells isolated from anti–IL-25–treated mice at 9 dpi, and subsequently stimulated with UV-irradiated RSV, secreted significantly less RSV-specific IL-5 and IL-13 than that secreted by mice treated with the isotype control (Fig. 6A). A small decrease in IL-4 was also detectable but was not statistically significant (data not shown). These data indicate that IL-25 plays a critical role in the differentiation of viral-specific Th2 cells. The attenuation of the Th2 cell responses by anti–IL-25 treatment also resulted in suppression of inflammation and histopathological changes in the lungs at 9 dpi. Both the number of mucus-secreting cells and eosinophils were significantly reduced (approximately 2-fold and 3-fold, respectively) compared with that for the isotype control treatment (Fig. 6B, 6C). Furthermore, the expression in the lung of IL-4 and IL-13 and the chemokine eotaxin-2 (factors that induce allergic inflammation) were all significantly reduced after anti–IL-25 treatment compared with that in the isotype control (Fig. 6D). By contrast, IFN-γ levels were unchanged, suggesting an exclusive effect of IL-25 on Th2-mediated rather than Th1-mediated immune responses. These data identify a crucial role for IL-25 in the induction of Th2 responses by RSV.

**IL-25-dependent Jagged1 expression on DCs is important for RSV-induced Th2 differentiation**

To investigate how the IL-25 signal might drive the generation of the Th2 phenotype, we examined IL-25 expression in the mediastinal lymph nodes. We were unable to detect mRNA or protein for this cytokine within these lymphoid tissues in any RSV-infected mice (data not shown). This suggested that IL-25 did not act directly to prime naive T cells within the lymph node compartment.
Next we examined if an indirect effect of IL-25 on Th2 differentiation occurred through a migrating intermediary cell, such as the DC. DC phenotype was characterized in the lymph nodes by the expression of the notch ligands associated with Th1 (dll4) and Th2 (Jagged1) polarization. The DCs expressed either ligand on the cell surface but not both on the same cell. Cells isolated from the lymph nodes at 4 dpi displayed a marked upregulation in the number of DCs expressing Jagged1 compared with the number expressing dll4 in NK-depleted mice compared with that in WT control mice (Fig. 7A). In particular, this was brought about by increased DC Jagged1 expression in the NK-depleted mice (Fig. 7A). This was not an effect of DC migration, as numbers of lymph node DCs remained unaltered between the groups (Supplemental Fig. 9A). Although there was an increase in the number of lung DCs in the NK-depleted mice, there was no change in the numbers expressing Jagged1 (Supplemental Fig. 9A, 9B). To determine a role for this elevated Jagged1 expression, this notch ligand pathway was inhibited using anti-Jagged1 mAb. The lymph node cells from mice at 4 dpi were cultured with anti-Jagged1 or an isotype control. At day 4, IL-25 protein expression was measured in lung sections by immunohistochemistry using FAST RED stain and images captured (original magnification ×40). The deep red color is specific for IL-25 protein. Scale is as depicted on the micrograph. Results represent two independent experiments with n ≥ 5 mice per group.

Discussion

Severe RSV infection has been linked to the development of childhood asthma and exacerbations of this disorder (2–4, 28). Recent clinical studies indicate that impaired NK cell function can readily occur during severe RSV infection (10, 12, 13), and as such these studies provide new evidence to suggest that this deficiency may underpin the association with asthma. Here we demonstrate the central importance of NK cells in maintaining appropriate protective immunity against viral infection and inhaled Ags. Furthermore, during RSV infection, we identify a critical role for NK cells and the production of IFN-γ for the prevention of deleterious viral-specific Th2 responses. To our knowledge, this study is the first to
demonstrate that NK cells negatively regulate the development of viral-specific Th2 responses, which has implications for how severe RSV infections may exert deleterious effects in promoting the pathogenesis of asthma.

The secretion of IFN-γ from NK cells is known to play a central role in the innate host defense response to viral infection (29). In this study, we demonstrate the critical importance of these factors, particularly early in the immune response to RSV infection, for limiting the long-term programming of viral-specific Th2 immune responses. NK cells and IFN-γ deficiency predisposed to the development of viral-specific Th2 effector cells and the onset of immune and pathological features of allergic airways disease (recruitment of eosinophils into the airways, mucus hypersecretion, and increased production of IgE). In an acute infection setting, we were unable to reconstitute these mice with NK cells due to the use of the depleting Ab. Notably, these Th2 cells persisted long term into memory and could be reactivated by secondary viral infection (42 d after primary infection) in the presence of normal NK cell numbers. Thus, factors that predispose to the impairment of NK cell and IFN-γ function during the acute phases of RSV exposure may have profound effects on the subsequent phenotype of immune response elicited long after infection. In the context of asthma, induction and re-enforcement of Th2 responses by RSV infection would significantly contribute to a mechanism of viral-induced pathogenesis. In a previous study, depletion of NK cells using a model of Bordetella pertussis infection has also been shown to enhance Th2 responses; however, the downstream regulatory pathways were not investigated (30).

Notably, during the early phase of RSV infection (2–4 dpi), the Th2 polarizing cytokine, IL-25, is produced from respiratory epithelial cells. Furthermore, the early production of IFN-γ from NK cells appears to play a critical role in suppression of IL-25 expression and in directing a protective antiviral immune response. The influx of NK cells at 4 dpi results in a pronounced increase in the levels of IFN-γ in the lung and the concomitant inhibition of IL-25 production from infected airway epithelial cells. However, in the absence of NK cells and IFN-γ, the IL-25 signal is not inhibited, and viral-specific Th2 cells are generated (between 4 and 9 dpi). Furthermore, the delivery of recombinant IFN-γ to the airways of NK cell-depleted mice infected with RSV, which suppressed the production of IL-25, also inhibited Th2 differentiation. To the authors’ knowledge, this paper is the first to establish an inverse relationship between IFN-γ and the regulation of IL-25 expression. This finding complements the observation that the expression of the IL-25R (IL-17BR) on airway smooth muscle cells is downregulated by IFN-γ and upregulated by TNF-α signaling (31).

The ability of IL-25 to promote the development of Th2 cells in models of allergic asthma and helminth worm infestation (32–36) is well established. However, a role for IL-25 in the development of viral-induced Th2 responses has not been described. By depleting IL-25 in mice where NK cell and IFN-γ function was impaired, we demonstrate a critical role of this cytokine in generating RSV-specific Th2 responses. This viral Th2 response was generated, at least in part, by the IL-25–dependent upregulation of the costimulatory molecule Jagged1 on lymph node DCs. The similar numbers of Jagged1-expressing DCs in the lungs of both treatment groups suggests that upregulation of Jagged1 in the absence of NK cells must occur either en route from the lungs to the lymph nodes or within the lymphoid tissue itself. When expressed on DCs, the notch ligands, Jagged1 and dll4, have been suggested to polarize naive CD4 T cells toward a Th2 or Th1 phenotype, respectively (37). Jagged1 expression on DCs can polarize both human (38) and mouse (39) T cells to the Th2 phenotype independently of IL-4. Furthermore, treatment of mice with Jagged1–Fc fusion protein has been shown to enhance inflammation in a model of allergic airways disease (39). Our data reveal an important role for IL-25 in the regulation of Jagged1 expression on mediastinal lymph node DCs that significantly contributes to the generation of viral-induced Th2 responses. Interestingly, Schaller et al. (40) demonstrated that during RSV infection, blockade of dll4 signaling directs the immune response away from a robust protective Th1 phenotype and enhances the generation of Th2 cytokines, mucus-secreting cells, and eosinophils. Thus, the latter study combined with our own results indicates that the appropriate control of notch ligand expression during RSV infection is critical to the programming of the adaptive CD4 T cell phenotype, which has significant implications for protective immunity. Under conditions where host defense pathways are impaired, RSV infection may induce prolonged production of epithelial-derived IL-25, which can alter DC costimulatory molecule expression to promote a Th2 differentiation program.

**FIGURE 7.** RSV-induced Th2 differentiation is dependent on DC Jagged1. A. At day 4, mediastinal lymph node cells were analyzed by flow cytometry for the ratio of Jagged1 to dll4 expressing CD11c+ MHC class II+ DCs (left panel) or percentage of Jagged1 expressing CD11c+ MHC class II+ DCs (right panel). Data represent the mean ± SEM. *p < 0.05 compared with WT control mice. B. At day 4, lymph node cells from NK-depleted RSV-infected mice were treated with anti-Jagged1 neutralizing Ab or an isotype control goat IgG and cultured for 3 d. Supernatants were analyzed for IL-13 and IFN-γ, Data represent the mean ± SEM, *p < 0.05 compared with WT control mice. C. At day 4, mediastinal lymph node cells were isolated from mice treated with anti–IL-25 or the isotype control and analyzed by flow cytometry for the ratio of Jagged1 to dll4 expressing CD11c+ MHC class II+ DCs (left panel) or percentage of Jagged1 expressing CD11c+ MHC class II+ DCs (right panel). Data represent the mean ± SEM. *p < 0.05; **p < 0.01 compared with isotype control. Results represent two independent experiments with n = 8 mice per group. ns, not significant.
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The precise way in which IL-25 modulates Jagged1 expression on lymph node DCs is not clear. IL-25 has been shown to act through its cognate receptor to drive allergic inflammatory processes by activating a subset of NK T cells and by inducing the differentiation of Th2 cells from naive CD4 T cells (23, 41). In our depletion model, we were unable to detect IL-25 within the lymph nodes of RSV-infected mice suggesting that these mechanisms did not operate. The IL-25R is also expressed on eosinophils, a population of alveolar macrophages, and DC-like cells (42–44). IL-25 may act directly on DCs within the airway mucus to upregulate Jagged1 expression before they migrate to the regional lymph nodes. IL-25 may have additional effects on as yet unidentified lung cells, which may also assist in promoting a Th2 polarizing environment.

Recently, IL-4–producing basophils have been shown to play a critical role in the differentiation of naive T cells into Th2 effector cells in response to specific allergens (45–47). Thus, we investigated if this cell may also contribute along with IL-25 in the generation of viral-specific Th2 responses. We observed that IL-4–producing basophils were specifically recruited to pulmonary lymph nodes in response to RSV infection, and this correlated with the development of Th2 cells. IL-4–secreting basophils have also been found in the parenchyma of STAT1−/− mice infected with RSV (48). However, depletion of basophils during exposure to RSV did not inhibit the development of viral-induced Th2 cells or features of allergic inflammation. Thus, a role for basophils may be limited to specific Th2 allergens, and their role in viral-induced Th2 differentiation may be redundant or they simply secrete IL-4 as a bystander effect of Th2 differentiation.

The mechanism whereby individuals become sensitized to normally innocuous environmental Ags and develop asthma remains largely unknown. Here we define a causal relationship between RSV infection and impairment of NK cell IFN-γ host defense mechanisms with sensitization to a bystander Ag leading to the generation of OVA-specific Th2 cells. Although Th2 cells were generated in response to NK cell depletion alone, the effect on polarization and inflammatory changes, both systemically and in the airways, was weak in comparison with the effect of concurrent infection with RSV. In this manner, NK cells appear to act as a tolerance “brake” in the absence of which sensitization to innocuous Ags can occur. The persistence of these viral-specific Th2 cells long-term (as we demonstrated) may mean that reexposure to RSV could potentially reactivate an allergic airways disease phenotype “asthma like” to the one we demonstrated may mean that reexposure to RSV could potentially reactivate an allergic airways disease phenotype “asthma like” to both the virus and any bystander allergen present at the time. One previous study suggested that NK cell depletion has limited impact on OVA-induced allergic airway inflammation. However, in this model, sensitization to OVA was achieved by intraperitoneal injection and without an inflammatory stimulus (i.e., a virus), which obfuscates any direct activation of innate immune pathways, such as NK cells, in the airways (49). Although we did not specifically clarify the mechanism underlying sensitization to OVA during RSV infection in the absence of NK cells, it is also likely to be linked to increased epithelial cell-derived IL-25 secretion arising from impaired NK cell IFN-γ production. Indeed, IL-25 has been shown to directly promote OVA-induced allergic airways disease in mouse models of asthma (33). Alternatively, the underlying mechanism may involve “collateral priming” through adaptive immune signals (50). In this scenario, RSV-specific Th2 cells would provide the polarizing signals, through secretion of IL-4, for the generation of the OVA-specific Th2 cells from naive bystander T cells.

The role of the innate immune system and epithelial cell-derived cytokines in the initiation of Th2 immunity and allergic responses is being increasingly recognized (21, 51). Despite this recent trend, our understanding of the mechanisms predisposing to viral-induced Th2 responses remains largely unclear. In this study, we demonstrate the importance of NK cells and IFN-γ as negative regulators of Th2 immunity to viral infection and foreign allergens. Impairment of these host defense mechanisms generates enhanced production of epithelial-derived IL-25 and the induction of Jagged1 expression on DCs leading to the development of RSV-induced Th2 responses and hallmark features of allergic inflammation. This defines for the first time a dynamic pathway by which the host epithelium interacts with innate immune cells to induce a viral-specific Th2 response. These findings also provide a potential mechanism whereby severe RSV infection may predispose to and/or exacerbate asthma in susceptible individuals; however, further human studies would be required to explore this relationship more thoroughly.

Disclosures

The authors have no financial conflicts of interest.

References


