Sustained inflammation and differential expression of interferons type I and III in PVM-infected interferon-gamma (IFNγ) gene-deleted mice

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Interferon gamma (IFNγ) has complex immunomodulatory and antiviral properties. While IFNγ is detected in the airways in response to infection with the pneumovirus pathogen, pneumonia virus of mice (PVM; Family Paramyxoviridae), its role in promoting disease has not been fully explored. Here, we evaluate PVM infection in IFNγ−/− mice. Although the IFNγ gene-deletion has no impact on weight loss, survival or virus kinetics, expression of IFNγ, IFNγR2 and IFNγ-stimulated 2–5′ oligoadenylate synthetases was significantly diminished compared to wild-type counterparts. Furthermore, PVM infection in IFNγ−/− mice promoted prominent inflammation, including eosinophil and neutrophil infiltration into the airways and lung parenchyma, observed several days after peak virus titer. Potential mechanisms include over-production of chemoattractant and eosinophil-active cytokines (CXCL1, CCL11, CCL3 and IL5) in PVM-infected IFNγ−/− mice; likewise, IFNγ actively antagonized IL5-dependent eosinophil survival ex vivo. Our results may have clinical implications for pneumovirus infection in individuals with IFNγ signaling defects.

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Introduction

The human pneumovirus pathogen, respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection among infants and children worldwide and has been recognized in the elderly (Borchers et al., 2013; Walsh 2011). A virus of the Family Paramyxoviridae, genus Pneumovirus, RSV is an enveloped single-stranded RNA virus that replicates in human alveolar macrophages and bronchial epithelial cells in vivo. While most disease is mild to moderate in nature, some infants are at high risk for severe disease, including those with bronchopulmonary dysplasia, immunodeficiency, premature birth and congenital heart disease; monoclonal antibody prophylaxis is available for these individuals (Shadman and Wald, 2011). Although not currently available, research and development is ongoing toward a safe and effective RSV vaccine (Rudraraju et al., 2013).

Pneumonia virus of mice (PVM) is a natural pathogen of rodent species that is of the same virus Family and genus as RSV. PVM undergoes robust replication in lung tissue, elicits a profound inflammatory response, and replicates the clinical picture of severe RSV infection in most inbred strains of mice (reviewed in Bem et al. (2011), Rosenberg and Domachowske (2008) and Dyer et al. (2012)). We have utilized the PVM infection model to explore the critical role of the proinflammatory cytokines in eliciting morbidity and mortality characteristic of severe RSV infection (Boville et al., 2003, 2004; Gabryszewski et al., 2011).

Interferon gamma (IFNγ) is a pleiotropic cytokine with complex immunomodulatory and antiviral properties. The biology and immunology of IFNγ (also known as Type II interferon) and its unique receptor, IFNγR, have been the subject of several excellent reviews (Schroeder et al., 2004; Durbin et al., 2013; Platanias 2005; Randall and Goodbourn, 2008; Schoenborn and Wilson, 2007; Akdis et al., 2011). Interestingly, IFNγ is not among the most prominent mediators detected in human RSV disease; levels have been reported in some (Fernandez et al., 2005; Garofalo et al.,...
2004; Juntti et al., 2013) but not all (Tabarani et al., 2013) studies of human infants infected with RSV. However, inbred mice have been used to investigate the role of IFNγ in modulating RSV disease. Among these studies, Culley et al. (2002) found that RSV challenge of newborn mice results in delayed IFNγ responses when compared to older mice, and that elevated levels of IFNγ resulting from delayed priming were associated with protection from severe disease. Similarly Lee et al. (2008) found that IFNγ present during primary RSV challenge of neonatal mice was crucial to maintain protection against development of airways hyperreactivity and lung histopathology upon secondary RSV challenge. Likewise, Empey et al. (2012) found that neonatal mice provided with recombinant IFNγ responded to RSV challenge with accelerated virus clearance in association with classical alveolar macrophage activation.

The role of IFNγ in promoting pathology has also been examined to some extent in primary PVM infection. For example, our group has shown that IFNγ is produced in response to primary PVM infection, but at levels that did not correlate directly with physical measures of lung dysfunction (Bonville et al., 2006). We have also found that IFNγ was not required for generation of adaptive immunity to PVM (Ellis et al., 2011). Just recently, Walsh et al. (2014) reported that IFNγ supported inflammation-related morbidity in PVM infection, although Frey et al. (2008) had previously examined PVM-induced morbidity in mice devoid of IFNγR, and found no differences between gene-deleted and wild type mice. Of note, all of the aforementioned studies were carried out using mice on the C57BL/6 background, a strain shown to be highly susceptible to PVM infection (Anh et al., 2006) to be a relatively resistant to PVM infection.

Given the importance of IFNγ in modulating immunity and immunopathology in virus infection, we embarked here on a more systematic evaluation of the impact of IFNγ gene-deletion on the BALB/c background, a strain shown to be highly susceptible to PVM infection (Anh et al., 2006). In addition to evaluating weight loss and survival, virus kinetics, and histopathology, we have determined a previously unappreciated role for IFNγ in regulating the expression of both IFNβ and IFNβ2/3 (Donnelly and Kotenko, 2010).

Results and discussion

IFNγ gene-deletion has no impact on survival or virus replication and clearance in response to PVM infection

IFNγ gene-deleted (IFNγ−/−) and wild-type BALB/c mice were infected with pneumonia virus of mice (PVM strain J3666; 0.033 to 3.3 TCID50 units in 50 µl inoculation volumes). As shown, the IFNγ gene-deletion had no impact on survival in response to PVM infection at any inoculation tested (Fig. 1A), and no impact on weight loss prior to the onset of mortality (Fig. 1B). Interestingly, IFNγ does protect against lethal infection with murine cytomegalovirus (Pomeroy et al., 1998), dengue virus (Fagundes et al., 2011; Costa et al., 2012) and murine gamma-herpesvirus (Lee et al., 2009; Tsai et al., 2011). In contrast, IFNγ signaling has no discernible impact on survival in response to vesicular stomatitis virus, Semliki forest virus, or influenza virus (Graham et al., 1993; Huang et al., 1993; Muller et al., 1994; van den Broek et al., 1995).

Our findings with PVM infection are similar to those in the latter group, and are also similar to those of Frey et al. (2008) who examined PVM strain 15 in IFNγ−/− mice, which are on the C57BL/6 background. Background strain can play a critical role in determining the impact of IFNγ signaling in acute infection; (Tsai et al., 2011) found C57BL/6 mice did not require IFNγ to control murine gamma-herpesvirus infection, while the same infection in BALB/c IFNγ−/− mice resulted in profound morbidity and mortality (Tsai et al., 2011; Lee et al., 2009).

Based on these results, we focused on the responses of both wild-type and IFNγ−/− mice to the minimal inoculum (0.033 TCID50/mouse in 50 µl inoculation volume) which generates a survivable infection in BALB/c mice. Both wild-type and IFNγ−/− mice that receive this inoculum undergo seroconversion to PVM antigens (data not shown); as documented previously, IFNγ signaling is not crucial for seroconversion and generation of anti-PVM IgG1 (Ellis et al., 2007). IFNγ was detected in lung tissue homogenates of wild-type mice on days 6–10 of infection; as anticipated, no immunoreactive IFNγ was detected at any time point in IFNγ gene-deleted mice (Fig. 1C). This response pattern was paralleled by production of CXCL9/MIG, a T cell chemoattractant whose expression is tightly regulated by IFNγ (Groom and Luster, 2011; Fig. 1D).

IFNγ gene-deletion also had no impact on virus replication and clearance (Fig. 1E). Both wild-type and IFNγ gene-deleted mice supported robust virus replication, with peak recovery at day 7 and diminished levels of virus detected thereafter. No virus was detected at day 21 post-inoculation. No differences in virus recovery were observed when comparing the wild-type and IFNγ gene-deleted mice at any time points evaluated.

IFNγ gene-deletion results in diminished expression of IFNβ and IFNβ2/3 in response to PVM infection

Immunoreactive IFNα was detected in the airways of PVM-infected wild-type and IFNγ−/− mice, with peak detected at day 7 of infection, correlating with peak virus recovery (Fig. 2A). Peak expression of transcripts encoding IFNβ (Fig. 2B) and IFNβ2/3 (IL28A/B; Fig. 2C) was also detected at day 7 of infection in both wild-type and IFNγ−/− mice. Expression of IFNβ2/3 has been determined previously in response to influenza virus infection (Jewell et al., 2010) and to PVM infection (Heinze et al., 2011). However, we show here that expression of transcripts encoding both IFNβ and IFNβ2/3 is significantly diminished (each 2–3 fold) in IFNγ−/− mice when compared to their wild-type counterparts (p < 0.001). We did detect immunoreactive IFNβ2/3 in both lung homogenates and bronchoalveolar lavage (BAL) fluid of IFNγ−/− mice. However, high background levels in uninfected tissue and BAL fluid, similar to what was reported previously by Heinze et al. (2011), precluded clear interpretation of these findings.

Expression of transcripts encoding IFNβ and IFNβ2/3 is also significantly diminished in PVM-infected type I interferon receptor (IFNAR1−/−) gene-deleted mice (Fig. 2D and E, (Muller et al., 1994)). Gene expression patterns have been documented for PVM infection in IFNAR1−/− mice (Garvey et al., 2005); in addition to diminished expression of numerous interferon-stimulated genes (ISGs), we observed overexpression of eotaxin-2 (CCL24) in mouse lung tissue in association with Th2-type pulmonary histopathology. Parallel to these findings, we observe diminished expression of 2–5′ oligoadenylate synthetase (Oas)1g, 2, and 3 in IFNγ−/− mice compared to their wild-type counterparts (Figs. 3A–C); differential expression of Mx1 does not reach statistical significance (Fig. 3D).

While there is substantial “cross-talk” via which IFNγ alters signaling initiated by IFNβ (and vice versa; reviewed in (Schroeder et al., 2004)), it is not immediately clear how IFNγ might limit the expression of IFNβ or IFNβ2/3 by more direct means. The promoters of IFNβ and of the better-characterized IFNα1 are both regulated by the coordinate actions of transcriptional factors NF-κB and interferon-regulated factors (IRFs)-3 and -7; although not yet fully characterized, the highly homologous promoter sequences of IFN2/3 share consensus binding sequences for these factors (Thomson et al., 2009; Durbin et al., 2013). IFNγ signaling may target one or both of these transcription factors, either directly or indirectly. There is already at least one example of this,
notably shown by Hu et al. (2006, 2009) who described IFNγ-mediated suppression of transcription factors CREB and AP-1, which ultimately leads to negative regulation of IL-10.

**IFNγ gene-deletion promotes pulmonary inflammation**

As shown in Fig. 4A, differential histopathology is observed at day 10, several days after peak virus titer at day 7. Lesions are more profound and diffuse in IFNγ−/− mice than in wild-type mice (Fig. 4B and C). Within the lung lesions in IFNγ−/− mice, we observe alveolar exudates with granulocytes and cell debris accompanied by a thickened interstitium. Compared to wild-type mice, IFNγ−/− mice also exhibit more abundant perivascular and peribronchiolar infiltrates, which also included granulocytes and mononuclear cells (Fig. 4D and E). Granulocytes (both eosinophils and neutrophils) are prominent components of the bronchoalveolar lavage fluid of IFNγ−/− mice (Fig. 5A and B). Similarly, significantly more eosinophils (SiglecF−CD11c+) and neutrophils (Gr1+) were detected in the lung tissue of IFNγ−/− than wild-type mice at these time points (Fig. 5C and D). In our recent work, we found that eosinophils recruited to the airways in response to Aspergillus fumigatus, but, interestingly, not in response to ovalbumin sensitization and challenge, become activated and release the specific granule protein, eosinophil peroxidase (EPX) in response to
subsequent challenge with PVM (Percopo et al., 2014). Here, in IFNγ−/− mice, eosinophils are recruited to the airways in response to PVM infection without prior priming, but they are not releasing substantial amounts of EPX (Fig. 5E).

Sustained inflammation, notably, sustained eosinophilic inflammation has been observed in the absence of IFNγ signaling in the setting of allergic (Coyle et al., 1996; Zhao et al., 2008) and infectious provocation (Wohllbeben and Erb, 2004). Several groups have explored the inflammatory response to RSV challenge in IFNγ−/− mice, although there remains no clear consensus on these results. Schwarze et al. (1999) documented substantial increases in eosinophils (5-fold) and moderate increases in neutrophils in lung tissue at 7 days after challenge with RSV in IFNγ−/− compared to wild-type BALB/c mice. Similarly, Boelen et al. (2002) reported eosinophil recruitment and accumulation at the bronchioles in response to RSV challenge of IFNγR−/− mice (129SvEv background strain). In contrast, Johnson et al. (2005) report that mice devoid of IFNγR (also on the 129SvEv background) exhibit inflammatory pathology that is indistinguishable from the wild-type, with profound inflammation observed only in mice devoid of both type I and type II IFN receptors or those devoid of the common signaling pathway (ie...Stat1−/− mice). As noted earlier with respect to the study of gammaherpesvirus infections, the specific background strain (Tsai et al., 2011), as well as age of the mice, preparation and size of the virus inoculum may have substantial impact on the results obtained.
Differential expression of chemoattractant cytokines

In addition to the aforementioned mechanisms that have been proposed to explain the more prominent inflammation observed in IFN$\gamma^{-/-}$ mice, we detect overproduction of both eosinophil and neutrophil chemoattractant cytokines in lung tissue of PVM-infected mice. Specifically, we find elevated levels of neutrophil chemoattractants CXCL1 and CCL3, the eosinophil chemoattractant CCL11 and the eosinophil-active cytokine, IL-5 in lung tissue of PVM-infected IFN$\gamma^{-/-}$ gene-deleted mice when compared to wild-type counterparts (Fig. 6). IL-5 was also detected in BAL fluid. Trace levels of GM-CSF, another eosinophil-active growth and pro-survival factor, were detected in lung homogenate and BAL fluid on day 7 only (data not shown). We have not defined the source of IL-5 in this setting, however it is intriguing to consider a role for virus-induced epithelial cytokines activating innate lymphoid cells (ILCs), notably IL-5-producing ILC2s in this setting (Kumar et al., 2014); similarly eosinophils themselves are a significant source of IL-5 (Rosenberg et al., 2013).

While the chemoattractant cytokines CXCL9, CXCL10, and CXCL11 are regulated directly by IFN$\gamma$-mediated signaling (reviewed in (Rauch et al., 2013; Groom and Luster, 2011)), other chemoattractant cytokines are differentially regulated in the absence of IFN$\gamma$ or its receptor; these findings are often invoked to explain differential leukocyte recruitment. For example, the neutrophil chemoattractants CXCL1 and CCL3 were reported as up-regulated in leukocytes recruited to the peritoneum of IFN$\gamma^{-/-}$ mice infected with Trypanosoma cruzi (Aliberti et al., 2001). Likewise, overexpression of CCL11 was observed in draining lymph nodes from IFN$\gamma^{-/-}$ mice immunized with interphotoreceptor retinoid binding protein (IRBP) to model autoimmune uveitis (Su et al., 2007). The specific connections and pathways linking IFN$\gamma$ signaling to these responses remain to be explored.

IFN$\gamma$ antagonizes eosinophil survival

While Ochiai et al. (1999) demonstrated that peripheral eosinophils express functional IFN$\gamma$ receptors, the role of IFN$\gamma$ in promoting specific eosinophil functions remains unclear. Our findings in Fig. 7A suggest that eosinophil survival may be limited in the presence of IFN$\gamma$, and thus eosinophil survival will be prolonged in IFN$\gamma$-deficient PVM-infected mice. Specifically, mouse eosinophils generated from bone marrow progenitors (Dyer et al., 2008) were maintained in culture for an additional 8 days in the presence of IL-5, IFN$\gamma$, or IL-5 and IFN$\gamma$ together, and ongoing survival was monitored. We found that IFN$\gamma$ in contrast to IL-5, does not support ongoing survival of eosinophils. Moreover, IFN$\gamma$ is not a neutral bystander, as it antagonized the pro-survival impact of IL-5 at the concentrations shown. This antagonism is wholly dependent on IFN$\gamma$ signaling, as it was not observed in mouse eosinophils generated from IFN$\gammaR^{-/-}$ progenitors (Fig. 7B). Clearly, eosinophils in tissue subjected to elevated but physiologically relevant levels IFN$\gamma$ (see Fig. 2C) may have reduced viability relative to their counterparts in IFN$\gamma^{-/-}$ mice. Thus, while Letuve et al. (2001) found that IFN$\gamma$ prolonged survival of human eosinophils ex vivo, our findings are more consistent with those of de Bruin et al. (2010) who found that mice with chronically-elevated levels of IFN$\gamma$ were devoid of eosinophils, and that eosinophilia and eosinophil hematopoiesis was suppressed in the bone marrow of these mice even in the presence of elevated levels of IL-5. While the mechanisms underlying these observations are not yet clear, these observations may likewise contribute to our understanding of the inflammatory responses in mice devoid of IFN$\gamma$. Equally intriguing, there are no published reports on the interactions of eosinophils with exogenous IFN$\gamma$. He et al. (2012) reported that human intestinal eosinophils express IFN$\gamma$, and that IFN$\gamma$ is released in response to inflammatory stimuli. However, the
impact of exogenous mediator on cell survival, and even the existence of receptors for IFNγ on human and/or mouse eosinophils remains to be determined.

It is interesting to reflect on these findings, given our understanding of patients devoid of interferon gamma receptor and thus unable to respond to interferon gamma (Casanova and Abel, 2002). These individuals are typically highly susceptible to infections with intracellular pathogens (e.g., mycobacteria). Although respiratory virus infection may be a comparatively limited concern in this patient group, heightened post-viral pulmonary inflammation observed among those individuals devoid of IFNγ signaling might be explored.

Materials and methods

Mouse and virus stocks

Wild-type BALB/c and C57BL/6 mice were purchased from Division of Cancer Therapeutics (DCT, National Cancer Institute, National Institutes of Health, MD). IFNγ gene-deleted (IFNγ−/−) mice (Dalton et al., 1993) on the BALB/c background are maintained by NDIT-Taconic contract and IFN−/−R1 gene-deleted mice were from Jackson Laboratories (Huang et al., 1993). CD2-IL5 transgenic mice (BALB/c background; (Dent et al., 1990)) are maintained on site; 8–14 week old mice were used in all experiments. IFNγ−/− mice (C57BL/6 background; (Muller et al., 1994)) were generated from heterozygotes as described previously (Garvey et al., 2005); RNA samples generated from PVM-infected (day 6 of infection) and uninfected mice for microarray analysis described in the aforementioned manuscript were utilized in this study. Mouse-passaged PVM strain J3666 is maintained as an in vivo passaged stock; virus titer was determined both by TCID50 (Percopo et al., 2011) and by a quantitative RT-PCR method (Gabryszewski et al., 2011). Mice received intranasal inocula (50 μL) of virus while under isoflurane anesthesia and were likewise anesthetized prior to sacrifice via cervical dislocation. All protocols were evaluated and approved as per the National Institutes of Allergy and Infectious Diseases Animal Study Protocol LAD 8E and carried out in accordance with the Institute’s Animal Care and Use Committee Guidelines.

Quantitative RT-PCR

Virus recovery was determined from cDNA generated from total RNA from mouse lung tissue by a dual standard curve qRT-PCR method that targets both the PVM small hydrophobic (SH) gene and mouse GAPDH as previously described (Gabryszewski et al., 2011). Detection of IFN-γ/3, IFN-γ, Mx1, Oas1g, Oas2 and Oas3 was carried out using the 20 × concentrated primer-probe set from ABI Assay by Design (Mm004204156_gH; Mm00439546_s1; Mm00487796_m1; Mm00726868_s1; Mm00460961_m1; Mm00460944_m1) used as per manufacturer’s instructions (ABI, Columbia, MD). Relative expression was determined via normalization to mouse GAPDH and by setting a single sample in the BALB/c wild-type day 0 set to 1.0.

Seroconversion

Anti-PVM IgG1 in mouse sera was detected by ELISA (Biotech Trading Partners, catalog #SMART-M12, El Cerrito, CA).

Histopathology

Prior to excision of lungs from the chest cavity, excess blood was removed by perfusion via the right ventricle with phosphate buffered saline (PBS), and the lungs were inflated trans-tracheally with 10% phosphate buffered formalin. The heart and the lungs were removed and fixed overnight in 10% phosphate buffered formalin at 4 °C. Samples were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (Histoserv, Germantown, MD).

Conclusions

Immunoreactive IFNγ is produced in response to PVM infection in mouse lung tissue. Although this has no discernible impact on virus replication/clearance or on survival in response to acute infection, we find that PVM-infected IFNγ gene-deleted mice express significantly less IFNγ and likewise significantly less IFNγ2/3 than do their wild-type counterparts. IFNγ gene-deletion results in more profound lung histopathology, which is detected several days after peak virus recovery. Persistent elevations of neutrophils and eosinophils are associated with differential expression of chemoattractant and eosinophil-active cytokines, and we show that IFNγ actively antagonizes eosinophil survival. The impact of this finding on human health, notably the role of heightened post-viral pulmonary inflammation observed among those individuals devoid of IFNγ signaling might be explored.

Fig. 4. IFNγ gene-deletion augments histopathology observed in response to non-lethal PVM infection. (A) Lung histopathology was scored as described by Hardy et al. (2009) as described in the Methods; shown are the means ± SEM, **p < 0.001, two-way ANOVA with Bonferroni’s multiple comparisons test. (B) Lung tissue from IFNγ−/− mice on day 10 post-inoculation, in which lesions are significantly more profound and diffuse than those seen in (C) lung tissue from wild-type mice (original magnifications, 5 × ); (D) likewise, more profound cellular infiltration is observed in lung tissue from PVM-infected IFNγ−/− mice when compared to a corresponding section from (E) PVM-infected wild-type mice (H&E staining, original magnifications, 40 × ); all mice inoculated with 0.033 TCID50 units PVM strain J3666.
The severity of pulmonary inflammation was scored according to a histopathologic scheme similar to that described by Hardy et al. (2009) as follows: a designation of severe (score 3) includes peribronchiolar and bronchiolar infiltrates and pneumonia that are diffuse and involving more than 50% of the lung tissue, moderate (score 2) includes focal peribronchiolar and bronchiolar infiltrates, and/or multifocal pneumonia present in 25 to 50% of the lung, and mild (score 1) consists in a single site or several localized lesions involving less than 25% of the lung; a fully normal appearance was given a score of 0. A value was assigned to each lung section and was used to calculate the total lung inflammation score; 3 sections were scored from each mouse.

**Bronchoalveolar lavage (BAL) and cell counts**

Neutrophils and eosinophils were evaluated in BAL fluid from PVM-infected mice using modified Giemsa stain (Diff-Quik; Fisher Scientific, Pittsburgh, PA). To prepare cells for staining, BAL fluids were subjected to centrifugation and re-suspended in 100 to 120 μL PBS plus 0.1% bovine serum albumin (BSA). Cells (10⁵ total) were
affixed onto slides using a Shandon Cytospin apparatus (Thermo-Electron, Pittsburgh, PA). Following staining and mounting of cells, 10 high-power fields were visually inspected by light microscopy.

Mouse eosinophil degranulation assay

This is ELISA for mouse eosinophil-specific eosinophil peroxidase (EPX) performed as described in Ochkur et al. (2012).

Lung cell preparation and flow cytometry

Lung tissue was harvested at the time points indicated and single cell suspensions prepared as described in Garcia-Crespo et al. (2013). Briefly, lung cells (∼10⁶) were first stained with LIVE/DEAD Fixable Yellow stain (Invitrogen, #L34959) in Hanks’ buffered saline solution (30 min, 4°C). Cells were then stained in PBS with 0.1% BSA in the presence of blocking anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA) for 60 min at 4°C with two different sets of antibodies: (i) anti-GR1-APC (BD Biosciences), anti-CD49b (DX5)-PE (BD Biosciences), anti-CD3-Alexa-700 (eBioscience, San Diego, CA), anti-CD4-FITC (eBioscience), anti-CD8-Pac-Blue (BioLegend, San Diego, CA), or (ii) anti-GR1-V450, anti-CD11c-Alexa-488, anti-SiglecF-PE (BD Biosciences), anti-MHC-II (I-A/I-E)-APC (eBioscience). Antibody-bound cells were washed twice in PBS-BSA 0.1% and fixed (4% paraformaldehyde/PBS). Data were analyzed using an LSRII flow cytometer (BD Biosciences) in conjunction with algorithms in FlowJo 9.6. Neutrophils and eosinophils were defined as GR1ʰ⁻/⁺CD3-CD4⁻CD8⁻DX5⁻ and SiglecF⁻/⁺CD11c⁻GR1ʰ⁻/⁺MHCII⁻ respectively. A minimum of 250,000 events was collected per sample; analyses are presented as percentage of live cells.

Proinflammatory cytokine expression

ELISA analysis was performed to quantify immunoreactive proteins using Quantikine and DuoSet® kits from R&D Systems (Minneapolis, MN) as per the manufacturer’s instructions. Sensitivities for the IL-5 and GM-CSF ELISAs are 7 and 5.8 pg/mL, respectively. Data points generated using lung tissue homogenates...
were normalized to total protein per sample, which was determined via BCA protein assay (Pierce, Rockford, IL).

**Generation and survival of mouse bone marrow eosinophils (bmEos)**

Bone-marrow-dervived eosinophils (bmEos) were generated from wild-type (C57BL/6) and IFN-R gene-deleted mice as described previously (Dyer et al., 2008). Cultures at day 12 contained 95–100% eosinophils, and became time point (t=0) for this study. From this point, cells were maintained in RPMI with 10% fetal calf serum and 10 mM glutamine with IL-5 (R&D Systems, 10 ng/mL), IFN-γ (Peprotech, 100 ng/mL) or both cytokines. Cell number and cell viability were monitored by hemocytometer counts with trypan blue staining in triplicate samples per experiment.

**Statistical analysis**

Data were analyzed using two-way ANOVA with Bonferroni’s multiple comparison tests, as appropriate. All statistical tests were included in the GraphPad Prism 6 software package (GraphPad Software, La Jolla, CA). Grubbs’ test was performed to detect outliers. All bar graphs indicate the mean ± SEM.

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