IL-15 complexes induce NK- and T-cell responses independent of type I IFN signaling during rhinovirus infection

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Rhinoviruses are among the most common viruses to infect man, causing a range of serious respiratory diseases including exacerbations of asthma and COPD. Type I IFN and IL-15 are thought to be required for antiviral immunity; however, their function during rhinovirus infection *in vivo* is undefined. In RV-infected human volunteers, IL-15 protein expression in fluid from the nasal mucosa and in bronchial biopsies was increased. In mice, RV induced type I IFN-dependent expressions of IL-15 and IL-15Rα, which in turn were required for NK- and $CD8^+$ T-cell responses. Treatment with IL-15–IL-15Rα complexes (IL-15c) boosted RV-induced expression of IL-15, IL-15Rα, IFN-γ, CXCL9, and CXCL10 followed by recruitment of activated, IFN-γ-expressing NK, $CD8^+$, and $CD4^+$ T cells. Treating infected $IFNAR1^{-/-}$ mice with IL-15c similarly increased IL-15, IL-15Rα, IFN-γ, and CXCL9 (but not CXCL10) expression also followed by NK-, $CD8^+$-, and $CD4^+$-T-cell recruitment and activation. We have demonstrated that type I IFN-induced IFN-γ and cellular immunity to RV was mediated by IL-15 and IL-15Rα. Importantly, we also show that IL-15 could be induced via a type I IFN-independent mechanism by IL-15 complex treatment, which in turn was sufficient to drive IFN-γ expression and lymphocyte responses.

INTRODUCTION

Infection with human rhinoviruses (RV) is the most common viral infection afflicting mankind. Although the nasal epithelium is the primary site of replication manifesting as the common cold, RV can also be detected in the lower airways[1,2] where it can cause severe and life-threatening exacerbations in patients with asthma, chronic obstructive pulmonary disease (COPD), and pneumonia.[3,4] Collectively, these diseases are responsible for significant morbidity and mortality and enormous health-care costs.[5]

Despite their significant involvement in respiratory diseases, surprisingly little is known about the role of cellular immune responses in RV pathogenesis. The involvement of natural killer (NK) cells and T cells during RV infection has been inferred from numerous studies demonstrating robust induction of lymphocyte-recruiting chemokines such as CXCL10 during infection.[6] Experimental infection studies in man have demonstrated infiltrating lymphocytes in nasal mucosa and bronchial biopsies associated with blood lymphopenia.[7] Characterization of blood leukocyte responses identified a significant reduction in $CD4^+$ T cells, suggesting that these are preferentially recruited to the site of RV infection.[8] More recently, mouse infection models have been developed allowing more detailed analyses of immune responses to RV. These have begun to confirm that RV can induce a range of lymphocyte-recruiting mediators associated with infiltration of NK cells and T cells to the lung.[9,10]

Interleukin-15 (IL-15) is a type I interferon (IFN)-induced cytokine that exerts antiviral effects by activation of NK cells and $CD8^+$ T cells.[11,12] IL-15 shares the IL-2Rβ- and common γ-chains of the IL-2 receptor,[13,14] but also binds its specific IL-15Rα-chain with much greater affinity.[15] Signaling occurs via a

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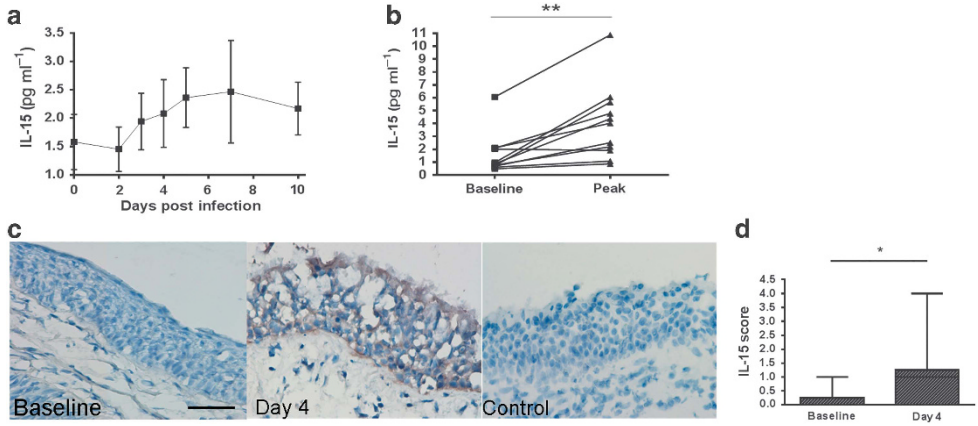


Figure 1 Experimental human RV infection induced IL-15 and associated Th1 and inflammatory mediators. Healthy volunteers were infected with RV-16 and levels of soluble mediators in the nasal mucosal fluid were measured by MSD platform and IL-15 in the epithelium of bronchial biopsies measured by IHC. (**a**) Levels of IL-15 protein in the nasal mucosa during infection. (**b**) Baseline and peak infection levels of IL-15. (**c**) Images of representative IHC staining for IL-15 protein in human bronchial biopsies at 14 days before infection (baseline), 4 days post infection with RV and normal mouse serum staining control. (**d**) IHC staining score for IL-15 in bronchial biopsies. Bar = 50 μm. IL-15 data are presented as mean ± s.e.m., baseline compared with infection peak data were analyzed by Mann–Whitney test. $^{**}P < 0.01$ and $^{*}P < 0.05$ as indicated. Differences were considered significant for all statistical tests at $P < 0.05$. All reported *P*-values are two-sided. IHC, immunohistochemistry; IL-15, interleukin-15; MSD, Meso-Scale Discovery; NS, not significant; RV, rhinovirus.

unique trans-presentation mechanism involving IL-15 expression by macrophages, dendritic cells (DCs), and epithelial cells where it is bound and stabilised by IL-15Rα and transported to the cell surface membrane for presentation to adjacent neighboring cells in *trans*.[16,17] During infection, IL-15 is critical for the activation, recruitment, and expansion of NK cells and $CD8^+$ T cells. NK cells are innate effector lymphocytes that can directly eliminate virally infected cells through the production of cytolytic enzymes such as granzymes and produce cytokines such as IFN-γ that regulate adaptive immune responses.[18,19] Direct type I IFN signaling is reported important for regulating NK-cell functions during viral infections.[20–22] NK-cell development and homeostasis is also dependent on IL-15 signaling as illustrated by the severe deficiency of NK cells in IL-$15^{-/-}$ and IL-$15R\alpha^{-/-}$ mice.[23,24] IL-15 and NK cells are also important for regulating adaptive T-cell responses during respiratory infections, with depletion of NK cells causing impaired $CD8^+$ T-cell responses against influenza and *M. tuberculosis*.[25,26]

We have previously used *in vitro* RV infection models involving bronchial epithelial cells and bronchoalveolar lavage (BAL) macrophages to identify deficient expression of type I IFN in asthma and COPD[27,28] and IL-15 in asthma,[29] identifying a potentially important link between IFN and IL-15 in the pathogenesis of RV-induced disease. To investigate the effect of loss of type I IFN-mediated responses *in vivo*, we used a mouse RV infection model observing severely impaired NK-cell and T-cell responses in type I IFN receptor knockout (IFNAR$1^{-/-}$) mice. Clearly type I IFN was critical for NK-cell responses to RV; however, the role of IL-15 was not studied.[10]

In the present study, we investigated the interaction between type I IFN-mediated responses, IL-15, and cellular immunity following RV infection *in vivo* in man and mouse. We found that RV infection induced IL-15 protein production in nasal and bronchial mucosa of healthy human subjects. We have provided novel *in vivo* data showing that RV-induced IL-15/IL-15Rα expression was mediated by type I IFN, which in turn was required for early expression of IFN-γ and recruitment of activated, IFN-γ-expressing NK cells and T cells. We have further demonstrated that exogenous delivery to the airways of biologically active IL-15 complexed with IL-15Rα (IL-15c) during RV infection in both wild-type (wt) and IFNAR$1^{-/-}$ mice boosted early lung expression of IFN-γ and the lymphocyte-recruiting chemokine CXCL9/MIG (monokine induced by gamma interferon) followed by lymphocyte recruitment.

RESULTS

RV infection-induced IL-15 in nasal and bronchial mucosa was associated with Th1 immunity

We employed a human experimental RV infection model recruiting healthy volunteers to determine whether RV infection induced IL-15 expression *in vivo*. In a group of 11 human subjects, we repeatedly sampled the nasal mucosal fluid before infection and up to 10 days after infection (**Figure 1a**). When baseline levels were compared with the peak infection levels for each subject, we observed a significant increase in IL-15 protein (**Figure 1b**). Viral load in nasal lavage was determined by PCR analysis of viral RNA levels. Although viral RNA- and IL-15 protein-levels typically peaked between day 2 and day 6 post infection, no statistically significant correlation for peak viral load with IL-15 was observed (data not shown).The lack of a clear relationship between peak IL-15 expression and viral load was likely due to the small number of subjects (11), which meant the study was not powered to quantify the dynamic interaction between IL-15 and viral load.

In a separate study also involving 11 healthy volunteers experimentally infected with the same RV-16 inoculum,[30] we used immunohistochemistry (IHC) to detect IL-15 protein expression in the lower airways (bronchial biopsies) at baseline

(2 weeks before infection) and 4 days after infection. IL-15 positivity was apparent on the apical surface of the epithelium and in cells directly below the epithelium (**Figure 1d**) and was significantly increased compared with uninfected subjects (**Figure 1e**).

IL-15 and IL-15Rα expression during RV infection

We used a mouse RV infection model to analyze *Il15* and *Il15rα* mRNA expression by BAL cells (**Figure 2a**) and lung tissue (**Figure 2b**), observing significantly increased levels compared with baseline and UV-inactivated RV-1B (UV-RV-1B)-dosed mice. Expression of mRNA for both molecules peaked at 1 day post infection (p.i.) returning to baseline levels by 4 days p.i. (**Figure 2a** and **b**). Upregulated expression of IL-15 and IL-15Rα proteins (measured as soluble IL-15-IL-15Rα complexes) in lung tissue similarly peaked at 1 day p.i. (**Figure 2c**). Further analysis of lung IL-15 protein by IHC revealed increased expression by bronchial epithelial cells and subepithelial DC-like cells in RV-infected mouse lungs (**Figure 2d**), which when scored was significantly higher than IL-15 expression in uninfected mice and mice dosed with UV-RV-1B (**Figure 2e**). A small increase in IL-15 protein following UV-inactivated virus was detected by IHC but was not apparent in lung homogenate using ELISA. This was likely due to differences in sensitivity of the two assays. The peak of IL-15 and IL-15Rα expressions coincided with peak viral loads in the lung at 1 day p.i., which remained elevated at 2 days p.i. and declined thereafter (**Figure 2e**).

Type I IFN receptor is required for IL-15 and IL-15Rα expression

Type I IFNs are reported to induce expression of IL-15 by macrophages, DCs, and epithelial cells,[11] and RV-induced expression of IFN-α and IFN-β in BALB/c mice (**Supplementary Figure S1** online) was associated with expression of IL-15 and IL-15Rα at 24 h p.i. We have previously shown that type I IFN receptor knockout (IFNAR1$^{-/-}$) mice have deficient type I and type III IFN production and NK-cell responses during RV infection; however, IL-15 expression was not assessed.[10] Therefore, we used these mice to determine whether type I IFN signaling was required for IL-15 expression during RV infection. Significantly attenuated *Il15* and *Il15rα* mRNA expression was observed in IFNAR1$^{-/-}$ mice compared with strain-matched wt mice (**Figure 3a**) with trends for reduction in IL-15 and IL-15–IL-15Rα protein complexes in lung tissue (**Figure 3b**).

IL-15 is critical for the NK-cell and CD8$^+$ T-cell responses during RV infection

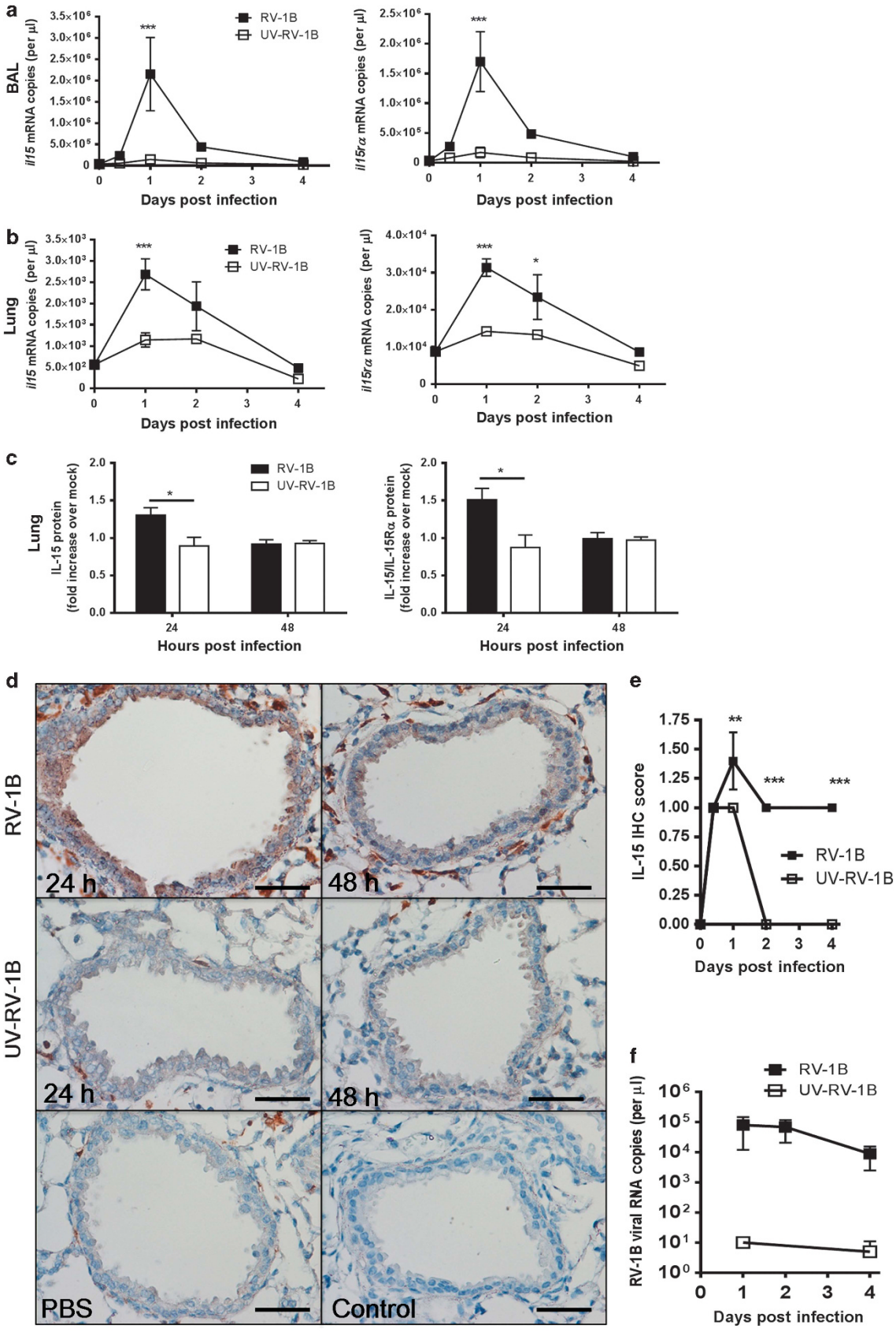
Next, we performed a time course analysis of NK-cell responses in the BAL and lung observing peak NK-cell accumulation at 2 days p.i., and peak numbers of NK cells expressing CD69, IFN-γ, and granzyme B were also detected at this time point (**Supplementary Figure S2**). NK-cell numbers declined to baseline by day 4 and day 7 in the BAL and lung, respectively, with levels of IFN-γ$^+$ NK cells remaining significantly elevated over controls at day 4 p.i. in the lung. UV-inactivated RV failed to induce NK-cell responses.

IL-15$^{-/-}$ and IL-15Rα$^{-/-}$ mice have a severe deficiency of NK cells. One option available to study IL-15-mediated NK-cell responses during RV infection was to transiently block IL-15 in the lung. We investigated the dependence of RV-induced NK cell responses on IL-15 by i.n. administration of an IL-15 neutralizing antibody (M96) at the time of infection in order to transiently block virus-induced lung IL-15 activity. To ensure the antibody did not greatly affect basal lymphocyte levels systemically, we assessed the lung and spleen in uninfected mice observing no significant change in total leukocyte cell numbers or percentage of CD4$^+$ and CD8$^+$ T cells, indicating that cells other than NK cells were not affected by antibody treatment in the absence of infection. In the spleen, the percentage of NK cells declined approximately 30% at 48 h, and in the lungs approximately 50% at 48 h after antibody treatment (**Supplementary Figure S3**).

Although antibody treatment modestly affected NK-cell baseline levels by 48 h post treatment, the administration of antibody at the time of infection, when NK-cell numbers were normal, permitted investigation into the role of RV-induced IL-15 on the accumulation and activation of NK cells. The presence of IL-15-neutralizing antibody at the time of infection resulted in a $>90\%$ and approximately 75% reduction in total NK-cell numbers in BAL and lung tissue, respectively, by 48 h after infection (**Figure 4a**). Similarly CD69$^+$, IFN-γ$^+$, or granzyme B$^+$ NK cells failed to accumulate with IL-15 neutralisation, further evidence of a critical role in pulmonary NK-cell responses during RV infection (**Figure 4b–d**). In association with these deficient NK-cell responses, *Ifnγ* gene expression in the lung was also significantly suppressed (**Figure 4e**).

As CD8$^+$ T-cell responses during RV infection have not been studied *in vivo*, we analyzed these cells and assessed the role of IL-15 using M96. We found that CD8$^+$ T-cell responses were also impaired in RV-M96-treated mice compared with controls (**Figure 5a**), analyzed at 48 h p.i., which was previously observed to be the peak of the CD8$^+$ T-cell response to RV infection in mice (data not shown). RV-M96-treated mice demonstrated significantly reduced numbers of total CD8$^+$ T cells, CD69$^+$ CD8$^+$ T cells, and IFN-γ$^+$ CD8$^+$ T cells compared with control-infected mice in the BAL. The same trend was observed in lung tissue, but statistical significance was only reached for reduced numbers of total CD8$^+$ T cells between RV-M96- and RV-PBS-treated mice (**Figure 5a**).

To further investigate the role of IL-15 during RV infection, we studied IL-15Rα knockout (IL-15Rα$^{-/-}$) mice, which have a severe deficiency of NK cells. We confirmed this and that RV infection could not induce NK-cell responses in these mice (**Supplementary Figure S4**). We next assessed total, CD69$^+$, and IFN-γ$^+$ CD8$^+$ T numbers in the airways and lung tissue during RV infection, observing that CD8$^+$ T-cell responses were also severely impaired in IL-15Rα$^{-/-}$ mice (**Figure 5b**) as was IFN-γ expression (**Figure 5c**). Transiently blocking IL-15 using M96 did not have a significant impact on the level of RV RNA in the lungs (**Figure 5d**). In contrast, by 24 h p.i. we did observe significantly increased RV RNA in the lungs of IL-15Rα$^{-/-}$ mice when compared with infected wt controls


a
BAL
il15 mRNA copies (per μl)
RV-1B
UV-RV-1B
***
Days post infection
il15rα mRNA copies (per μl)
***
Days post infection
b
Lung
il15 mRNA copies (per μl)
RV-1B
UV-RV-1B
***
Days post infection
il15rα mRNA copies (per μl)
***
*
Days post infection
c
Lung
IL-15 protein (fold increase over mock)
RV-1B
UV-RV-1B
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Hours post infection
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d
RV-1B
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24 h
48 h
24 h
48 h
PBS
Control
e
IL-15 IHC score
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***
RV-1B
UV-RV-1B
Days post infection
f
RV-1B viral RNA copies (per μl)
RV-1B
UV-RV-1B
Days post infection

(**Figure 5e**). We noted that viral load was higher in B6.129 mice (control for IL-15Rα$^{-/-}$ mice) compared with BALB/c mice used in the anti-IL-15 antibody-blocking studies. Therefore, differences in mouse strain susceptibility to viral infection may have also contributed to the greater apparent effect of IL-15 receptor deficiency on lung viral load.

Exogenous IL-15–IL-15 receptor complexes do not require type I IFN to induce IFN-γ and lymphocyte recruitment

RV infection studies with models of IL-15 deficiency revealed that IL-15 was required for IFN-γ expression and lymphocyte activation and recruitment (**Figures 4** and **5**). We hypothesized that supplementing bioactive IL-15 complexed to IL-15Rα (IL-15c) should boost these responses. The role of type I IFN in this process was also investigated using IFNAR1$^{-/-}$ mice. We have previously reported that IFNAR1$^{-/-}$ mice fail to recruit and activate NK cells in response to RV infection,[10] which was confirmed for this study (**Supplementary Figure S5**). IL-15c treatment alone (in the absence of infection) did not affect baseline lung NK cell or CD4$^+$ and CD8$^+$ T-cell numbers or activation status in either wt or IFNAR1$^{-/-}$ mice. In RV-infected wt mice, IL-15c treatment significantly boosted NK-cell responses over that induced by infection alone in the BAL (**Figure 6a**) and lung (**Figure 6b**) as indicated by increased total, CD69$^+$, IFN-γ$^+$, and granzyme B$^+$ NK-cell numbers. Furthermore, administering IL-15c to IFNAR1$^{-/-}$ mice completely restored NK-cell responses to RV infection to levels similar to those observed in IL-15c-treated wt mice. Similarly, IL-15c treatment also significantly boosted RV-induced BAL (**Figure 6c**) and lung (**Figure 6d**)-activated CD8$^+$ T-cell responses in wt mice. In IFNAR1$^{-/-}$ mice, IL-15c-stimulated CD8$^+$ T-cell responses were comparable to those observed in IL-15c-treated wt mice. CD4$^+$ T-cell responses were also boosted with IL-15c treatment to similar levels in both wt and IFNAR1$^{-/-}$ RV-infected mice compared with infection alone in the BAL (**Figure 6e**) and lung (**Figure 6f**). These results demonstrate that IL-15 can augment RV-induced lymphocyte responses independently of type I IFN signaling.

IL-15c increased IL-15 and type I and type II IFN responses

To determine which IL-15-stimulated responses do not require type I IFN signaling during RV infection, we assessed expression of antiviral/Th1-associated molecules in wt and IFNAR1$^{-/-}$ mice following IL-15c treatment with RV infection. IL-15c enhanced both *Il15* and *Il15rα* lung gene expression in wt and

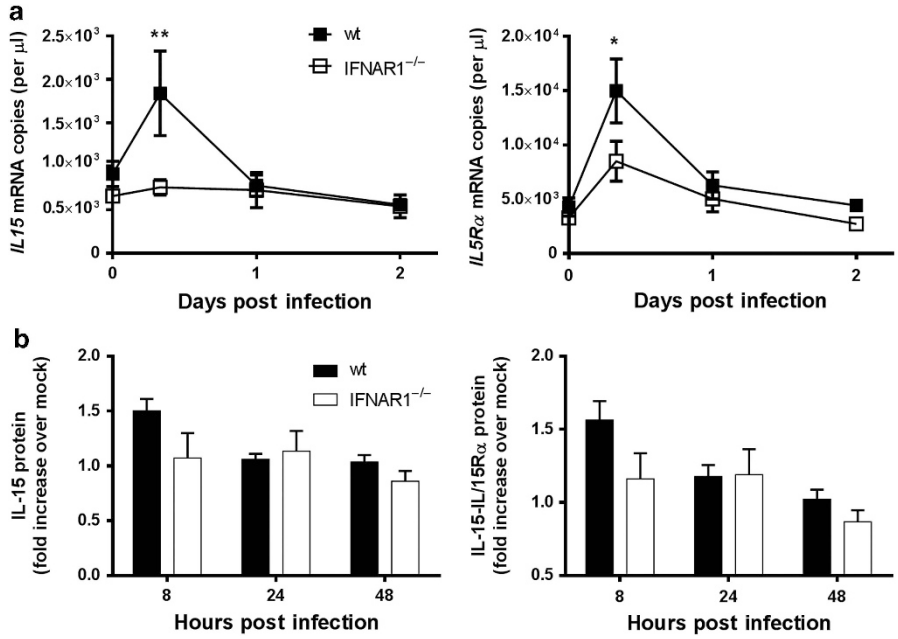

Figure 3 Type I IFN receptor is necessary for RV-induced IL-15 and IL-15Rα. IFNAR1$^{-/-}$ and wt strain-matched controls were either infected i.n. with 5×10^6 $TCID_{50}$ of RV-1B or dosed with PBS (mock/0 h post infection). (**a**) Levels of *Il15* and *Il15rα* mRNA were measured by qPCR in lung tissue. (**b**) IL-15 and soluble IL-15-IL-15Rα complex (IL-15c) protein levels in lung homogenate expressed as fold increase over mock infected. All data are representative of three independent experiments, $n = 4$–6 mice per group. Data were analyzed by two-way ANOVA, $^{**}P < 0.01$ and $^{*}P < 0.05$ as indicated, all data are expressed as mean ± s.e.m. ANOVA, analysis of variance; IHC, immunohistochemistry; IL-15, interleukin-15; IFN, interferon; RV, rhinovirus.

Figure 2 Rhinovirus-induced IL-15 and IL-15Rα gene and protein expression. BALB/c mice were either infected i.n. with 5×10^6 $TCID_{50}$ of RV-1B or dosed with inactivated UV-RV-1B or PBS (mock infected/0 days post infection). (**a**, **b**) Levels of *Il15* and *Il15rα* mRNA were measured by qPCR in BAL cells (**a**) and lung tissue (**b**). (**c**) IL-15 and soluble IL-15-IL-15Rα complex (IL-15c) protein levels in lung homogenate expressed as fold increase over mock infected. (**d**) Images of representative IHC staining for IL-15 protein in lung sections at 24 h and 48 h post infection (RV-1B) or treatment with UV-inactivated virus (UV-RV-1B), mock infection (PBS) and normal goat serum staining control. Original magnification, × 400. Bar = 50 μm. (**e**) Time course of IL-15 staining based on IHC score. (**f**) Time course of RV-1B viral load measured by qPCR in lung tissue. All data are representative of two to three independent experiments, $n = 4$–6 mice per group. Data were analyzed by two-way ANOVA, $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ as indicated, all data are expressed as mean ± s.e.m. ANOVA, analysis of variance; IHC, immunohistochemistry; IL-15, interleukin-15; IFN, interferon; RV, rhinovirus.

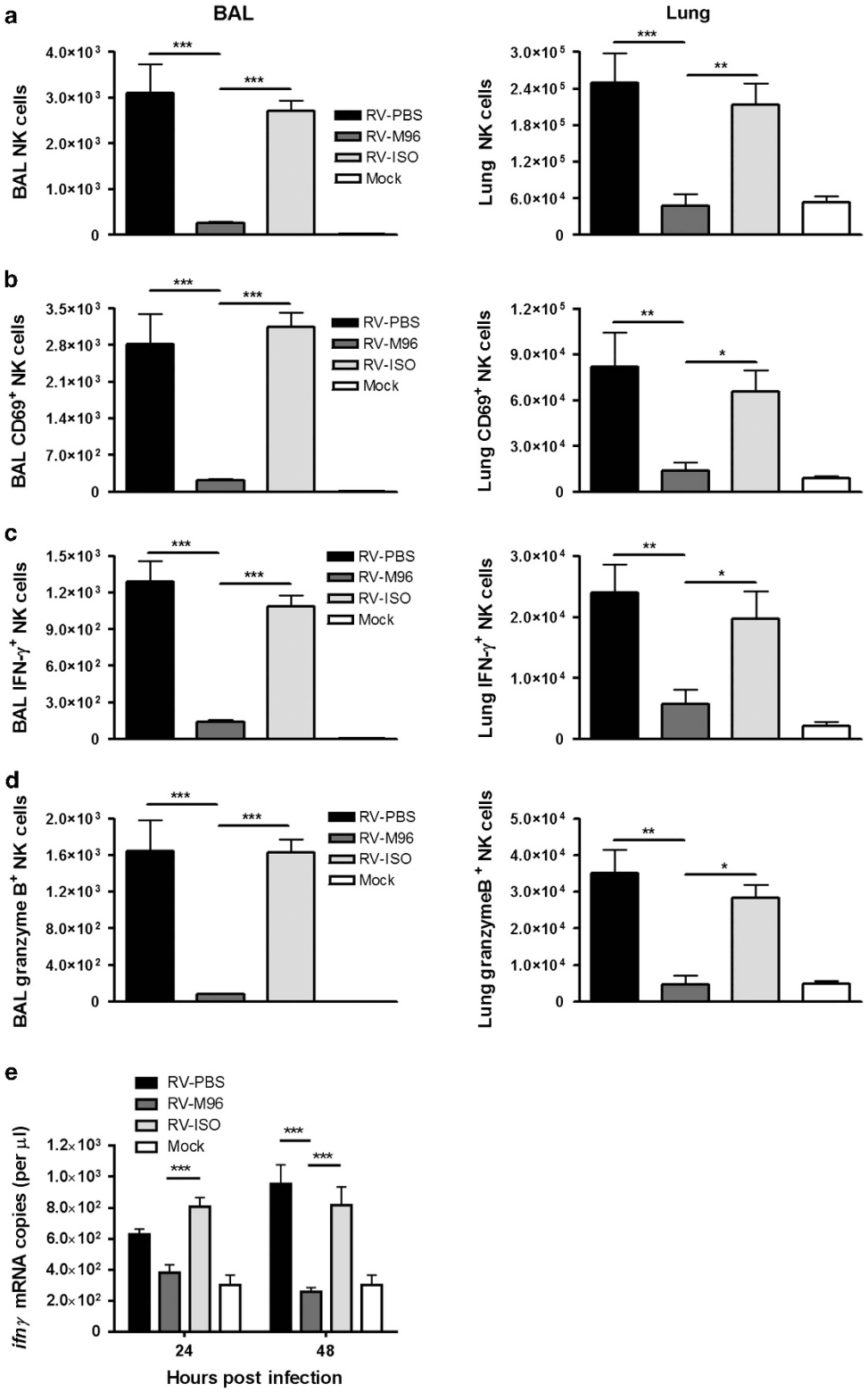

Figure 4 Rhinovirus-induced NK-cell responses were dependent on IL-15 signaling. BALB/c mice were either infected i.n. with 5×10^6 $TCID_{50}$ of RV-1B or dosed with PBS in addition to an IL-15-neutralizing antibody (M96), isotype control antibody, or PBS at the time of infection. (**a**–**d**) Flow cytometry was used to determine the total number of NK cells ($CD3^-$ $NKp46^+$) (**a**), $CD69^+$ NK cells (**b**), IFN-γ^+ NK cells (**c**), and granzyme B^+ NK cells (**d**) in the BAL and lung tissue, at 48 h post infection. (**e**) Levels of *Ifnγ* mRNA were measured by qPCR in lung tissue. All data are representative of two independent experiments, $n = 4$–7 mice per group. Data were analyzed by two-way ANOVA, $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ as indicated, all data are expressed as mean ± s.e.m. ANOVA, analysis of variance; IHC, immunohistochemistry; IL-15, interleukin-15; IFN, interferon; NK, natural killer; RV, rhinovirus.

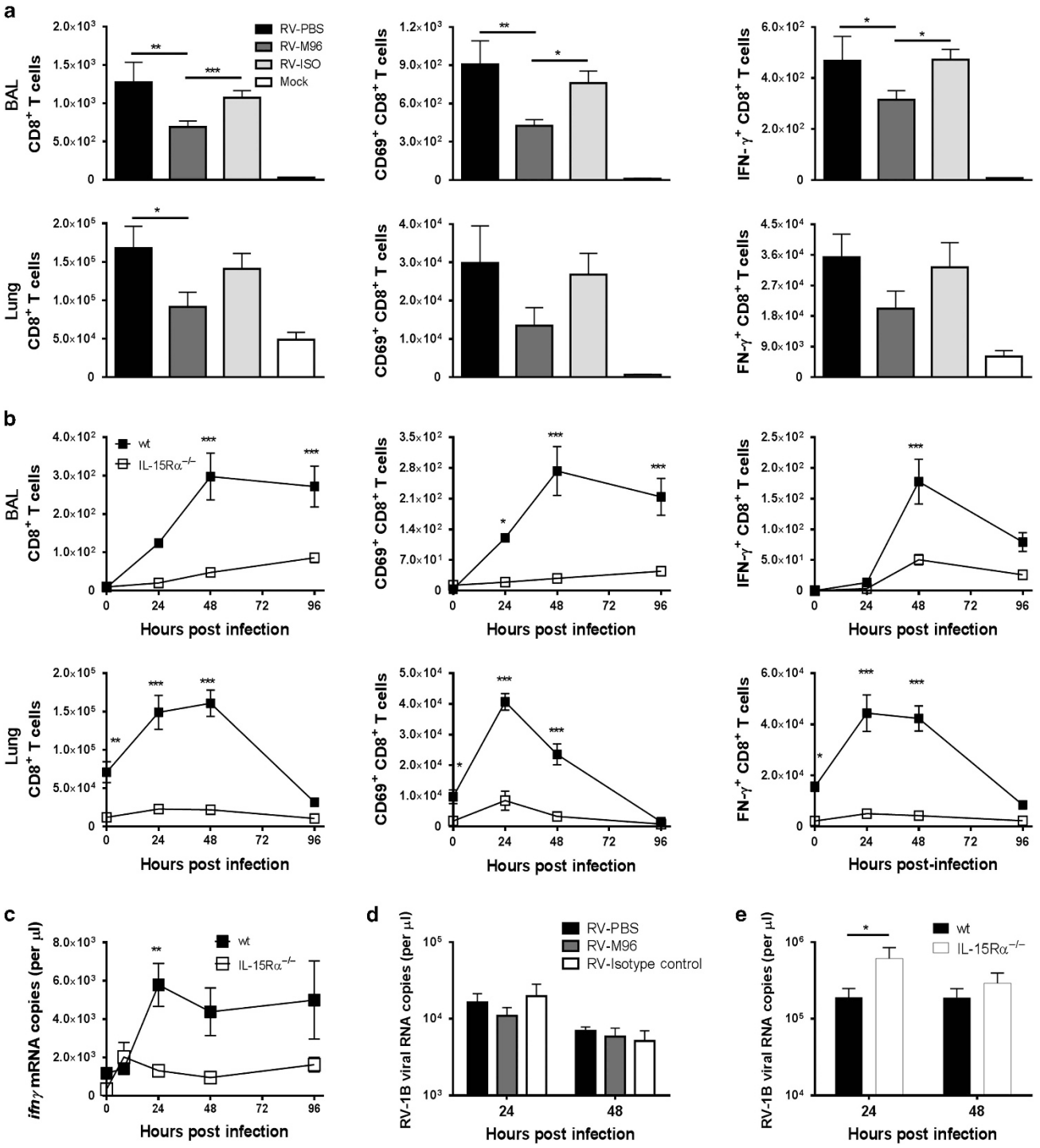

Figure 5 IL-15 signaling was important for CD8$^+$ T-cell responses during rhinovirus infection. BALB/c mice were either i.n. infected with 5×10^6 $TCID_{50}$ of RV-1B or dosed with PBS in addition to an IL-15-neutralizing antibody (M96), isotype control antibody or PBS at the time of infection. Alternatively, IL-15Rα$^{-/-}$ and strain-matched control wt mice were either i.n. infected with 5×10^6 $TCID_{50}$ of RV-1B or dosed with PBS (mock/0 h post infection). Flow cytometry was used to determine the total number of CD8$^+$ T cells (CD3$^+$ CD8$^+$), CD69$^+$ CD8$^+$ T cells, and IFN-γ$^+$ CD8$^+$ T cells in the BAL and lung of (**a**) M96-treated BALB/c mice at 48 h post infection and (**b**) IL-15Rα$^{-/-}$ or wt mice at the indicated time points after treatment. (**c**) Levels of *Ifnγ* mRNA were measured by qPCR in lung tissue. Levels of RV-1B viral RNA in lung tissue were measured by qPCR to determine the viral load in (**d**). M96-treated BALB/c mice and (**e**) IL-15Rα$^{-/-}$ and wt mice. All data are representative of two to three independent experiments, $n = 4$–7 mice per group. Data were analyzed by two-way ANOVA, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ as indicated, all data are expressed as mean ± s.e.m. ANOVA, analysis of variance; IHC, immunohistochemistry; IL-15, interleukin-15; IFN, interferon; RV, rhinovirus.

IFNAR1$^{-/-}$ mice (**Figure 7a**). There was a trend for increased *Ifnβ* gene expression in wt and IFNAR1$^{-/-}$ mice with IL-15c treatment (**Figure 7b**), and this corresponded with significantly increased BAL IFN-β protein levels in IFNAR1$^{-/-}$ mice but no increase was detected in wt mice (**Figure 7b**). IFN-α was poorly induced during RV infection in IFNAR1$^{-/-}$ mice in contrast to

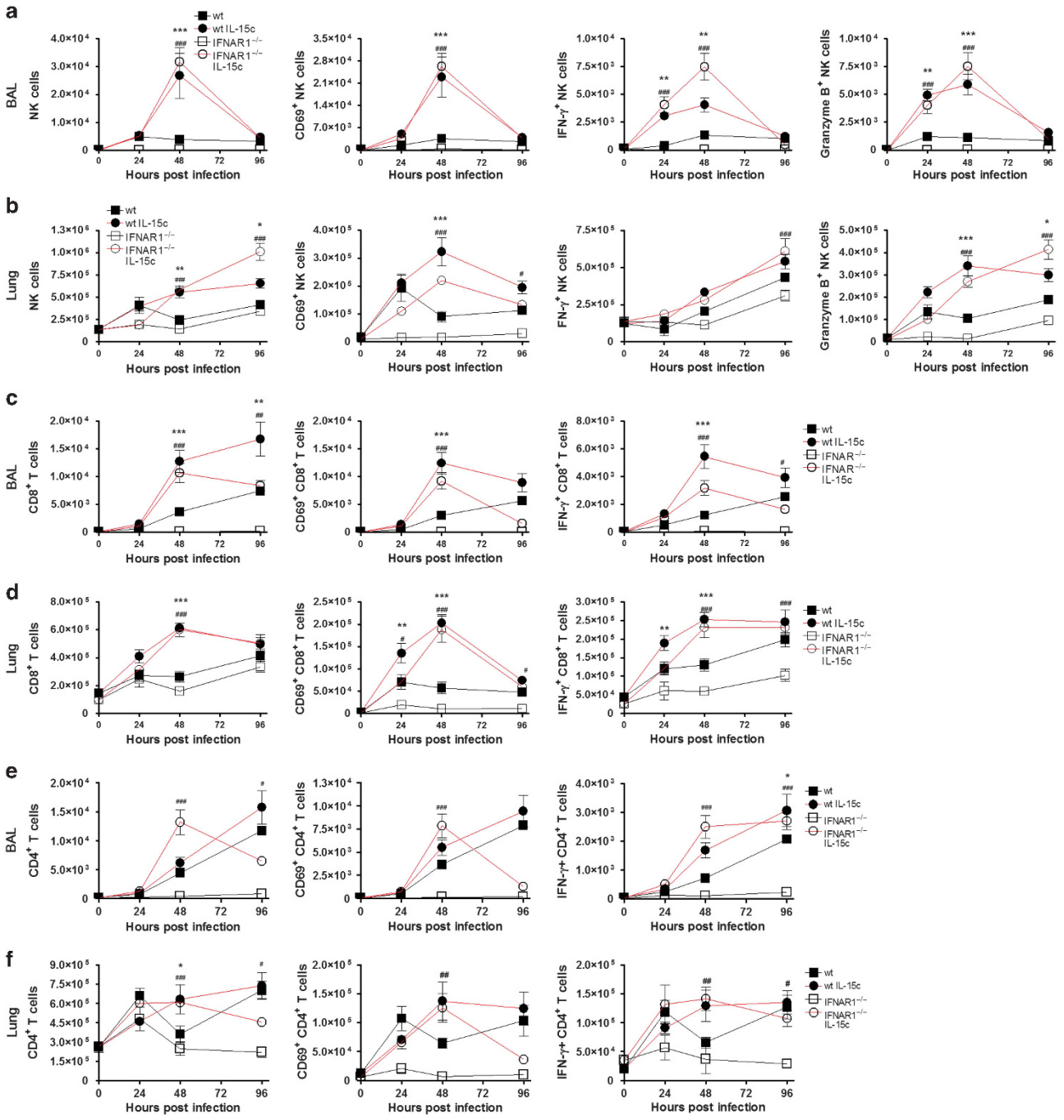

Figure 6 IL-15 complex restored NK-cell, $CD8^+$ and $CD4^+$ T-cell responses in the absence of type I IFN receptor signaling. $IFNAR1^{-/-}$ and strain-matched wt control mice were either infected with 5×10^6 $TCID_{50}$ of RV-1B i.n. or dosed with PBS (mock/0 h post infection) in the absence or presence of IL-15-IL-15Rα complex (IL-15c). (**a**–**f**) Flow cytometry was used to determine the time course of total numbers of NK cells ($CD3^-$ $NKp46^+$), $CD69^+$ NK cells, IFN-γ^+ NK cells, and Granzyme B^+ NK cells in the BAL (**a**) and lung (**b**); $CD8^+$ T cells ($CD3^+$ $CD8^+$), $CD69^+$ $CD8^+$ T cells and, IFN-γ^+ $CD8^+$ T cells in the BAL (**c**) and lung (**d**); and $CD4^+$ T cells ($CD3^+$ $CD4^+$), $CD69^+$ $CD4^+$ T cells and IFN-γ^+ $CD4^+$ T cells in the BAL (**e**) and lung (**f**) at the indicated time points post infection. All data are representative of three independent experiments, $n = 4$–6 mice per group. Data were analyzed by two-way ANOVA, $^{\#\#\#/***}P < 0.001$, $^{\#\#/**}P < 0.01$ and $^{\#/*}P < 0.05$ as indicated, all data are expressed as mean ± s.e.m. * refers to wt vs. wt IL-15c groups and $^{\#}$refers to $IFNAR1^{-/-}$ vs. $IFNAR1^{-/-}$ IL-15c groups. ANOVA, analysis of variance; IHC, immunohistochemistry; IL-15, interleukin-15; IFN, interferon; RV, rhinovirus.

wt mice. Even so, IL-15c treatment was able to significantly increase the detectable level of BAL IFN-α protein in $IFNAR1^{-/-}$ mice. For wt, mice despite robust induction, IL-15c actually reduced the level of IFN-α. IL-15c treatment significantly increased IFN-λ mRNA expression in both RV-infected wt and $IFNAR1^{-/-}$ mice; however, corresponding increases in BAL IFN-λ protein were only significant in $IFNAR1^{-/-}$ mice (**Figure 7b**). Furthermore, despite similar

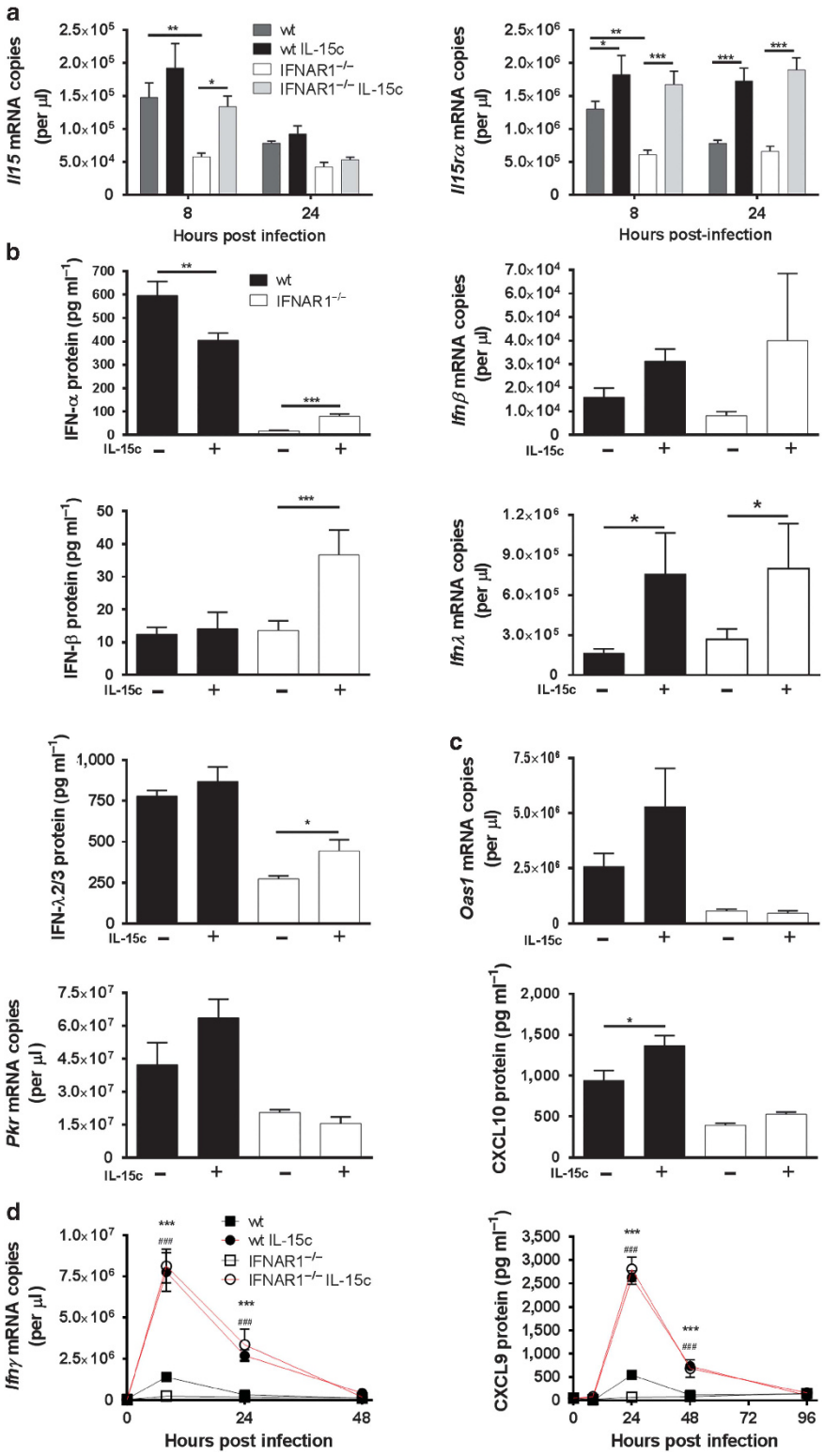

Figure 7 IL-15c administration during rhinovirus infection boosts expression of IL-15, IL-15Rα, type I, II, and III IFNs, and ISGs. IFNAR1$^{-/-}$ and strain-matched control wt mice were either infected i.n. with 5×10^6 $TCID_{50}$ of RV-1B or dosed with PBS (mock/0 h post infection) in the presence or absence of IL-15c. (**a**) Levels of *Il15* and *Il15rα* mRNA were measured by qPCR in lung tissue at 24 and 48 h post infection. (**b**) Levels of IFN-α, IFN-β, and IFN-λ2/3 protein in BAL fluid were measured by ELISA and *Ifnβ* and *Ifnλ* mRNA in lung tissue by qPCR at 24 h post infection. (**c**) Expression of ISGs *Pkr* and *Oas1* mRNA in lung tissue and CXCL10 protein in BAL 24 h post infection. (**d**) Lung *Ifnγ* mRNA and CXCL9 protein in BAL fluid 24 h post infection; * refers to wt vs. wt IL-15c groups and # refers to IFNAR1$^{-/-}$ vs. IFNAR1$^{-/-}$ IL-15c groups. All data are representative of two to three independent experiments, $n = 4$–6 mice per group. Data were analyzed by two-way ANOVA, $^{\#\#\#/***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ as indicated, all data are expressed as mean ± s.e.m. ANOVA, analysis of variance; IHC, immunohistochemistry; IL-15, interleukin-15; IFN, interferon; RV, rhinovirus.

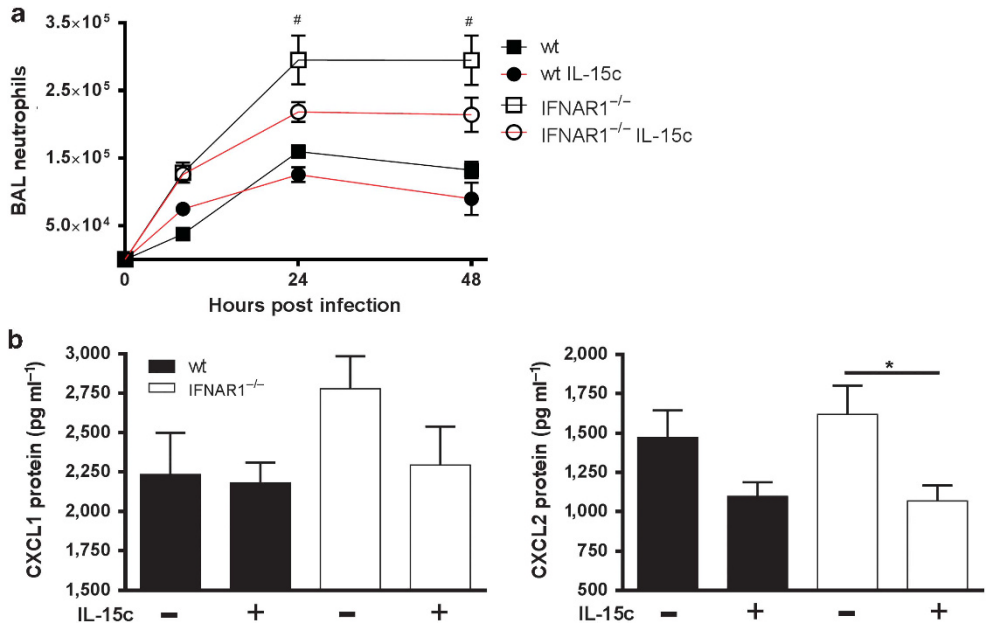

Figure 8 IL-15c suppressed exacerbated neutrophilic inflammation and associated chemokine expression in RV-infected type I IFN receptor-deficient mice. IFNAR1$^{-/-}$ and wt mice were either infected i.n. with 5×10^6 $TCID_{50}$ of RV-1B or dosed with PBS (mock/0 h post infection) in the absence or presence of IL-15c. (**a**) Timecourse of neutrophils in the BAL was assessed by differential cell counts, at the indicated time points after treatment; # refers to IFNAR1$^{-/-}$ vs. IFNAR1$^{-/-}$ IL-15c groups. (**b**) Neutrophil chemokines CXCL1 and CXCL2 were analyzed in BAL fluid 8 h post infection by ELISA. All data are representative of two to three independent experiments, $n = 4$–6 mice per group. Data were analyzed by two-way ANOVA, $^{\#/*}P < 0.05$ as indicated, all data are expressed as mean ± s.e.m. ANOVA, analysis of variance; IHC, immunohistochemistry; IL-15, interleukin-15; IFN, interferon; RV, rhinovirus.

levels of IFN-λ mRNA detected in corresponding treatment groups of wt and IFNAR1$^{-/-}$ mice, greater levels of protein was observed in wt mice.

We have previously shown full induction of the ISGs 2′-5′-oligoadenylate synthetase 1 (*Oas1*), protein kinase R (*Pkr*), and CXCL10 during RV infection required expression of type I IFN receptor.[10] IL-15c treatment augmented RV-induced expression of (*Oas1*) mRNA, (*Pkr*) mRNA, and CXCL10 protein in wt mice; however, statistical significance was only reached for increased CXCL10 expression (**Figure 7c**). IL-15c treatment also significantly increased lung *Ifnγ* gene expression at 8 h p.i., which was followed by significantly increased production of CXCL9 at 24 h p.i. in wt and IFNAR1$^{-/-}$ mice to equal levels (**Figure 7d**). These data show that IL-15c treatment greatly boosted expression of IFN-γ and IFN-γ-stimulated chemokines during RV infection via a type I IFN-independent mechanism.

IL-15c treatment reduced airway neutrophilic inflammation

A feature of the response to RV infection in human and mouse models is neutrophilic inflammation and this is exacerbated by IFN deficiency.[10] We compared airway neutrophilia following RV infection observing a significant increase in IFNAR1$^{-/-}$ mice compared with wt as previously reported. IL-15c treatment reduced the numbers of neutrophils in the airways of IFNAR1$^{-/-}$ mice (**Figure 8a**). This was associated with reduced expression of the neutrophil-recruiting chemokines CXCL1 and CXCL2 at 8 h p.i. (**Figure 8b**), and by 24 h p.i. levels had declined to baseline in all groups (data not shown).

DISCUSSION

Despite their dominant role in respiratory diseases globally, much is still unknown about the immunopathogenesis of RV infections. In this study, we first used a human experimental RV infection model observing increased IL-15 protein expression in the upper and lower respiratory tract. The nasosorption method of repeated sampling of the upper respiratory tract provided undiluted samples of nasal mucosal lining fluid that allowed detection of numerous immune mediators. This enabled us to detect IL-15 protein (as well as many other immune mediators) directly in the mucosa of both the upper and lower airways during RV infection, a first for RV and indeed for any respiratory virus.

RVs primarily replicate in nasal epithelial cells of the upper respiratory tract and cause the common cold. RVs can also infect the lungs but usually only cause disease when infection exacerbates a pre-existing respiratory condition such as asthma[30] or COPD.[28] To study 'normal' host immunity to RV infection in the absence of pre-existing respiratory disease, we employed a mouse RV infection model. This involved delivery of a high dose of RV-1B that, while not designed to model human disease *per se*, is widely used to study immunity to RV as it generates robust and reproducible inflammatory and antiviral immune responses in commonly used mouse strains including Balb/c,[9] C57BL/6,[31] and B6.129,[10] which were the strains used in this study. For all of these mouse strains, it was reported that RV infection induced an acute neutrophilic response accompanied by expression of neutrophil-recruiting chemokines, proinflammatory cytokines, and interferons. This

was followed by expression of lymphocyte-recruiting chemokines, resolution of neutrophilic inflammation, and recruitment of lymphocytes to the lungs.

Investigation of the role of IL-15 is complicated by constitutive expression and requirement for NK-cell homeostasis. This potentially contributed to the modest induction levels of IL-15 protein expression observed during RV infection in human and mouse models. We employed two strategies to block IL-15 activity during RV infection. The first involved lung delivery of an IL-15-neutralizing antibody to transiently block IL-15 during infection. In uninfected mice, this eventually caused a 50% reduction in lung NK cells after 2 days. During infection, IL-15 neutralisation (at the time of infection so NK-cell numbers intact) almost completely ablated lung tissue and airway NK-cell responses providing direct evidence that IL-15 is required for acute NK-cell responses during RV infection. We also studied IL-15Rα$^{-/-}$ mice that have a constitutive severe deficiency of NK cells and lower numbers of CD8^{+} T cells in the lungs at baseline. IL-15Rα$^{-/-}$ mice had severely deficient CD8^{+} T-cell responses, demonstrating that IL-15 is necessary for recruitment of activated IFN-γ-expressing CD8^{+} T cells. Reduced CD8^{+} T-cell responses were also observed with IL-15 neutralisation by an antibody during infection; however, the reduction was modest.

Despite having profound effects on host immune responses, IL-15 activity only modestly influenced viral replication. IL-15Rα$^{-/-}$ mice had a transient increase in viral load. Anti-IL-15 antibody treatment had no effect on levels of lung viral RNA. This difference is associated with the different degrees of CD8^{+} T-cell deficiency discussed above. Little is known about the kinetics of RV replication in the human lung, as it is technically difficult to repeatedly sample individual subjects during infection.[32] In the absence of this information, it is difficult to interpret lung replication data in the mouse. Also the dose of virus required to induce robust pulmonary immune responses in mice is high and did not reflect a natural infection in humans, which usually does not induce substantial lower respiratory tract inflammation. Thus, RV replication data in the mouse should be interpreted with caution particularly in the absence of good human *in vivo* lung infection data to support such analyses.

This is the first *in vivo* demonstration of type I IFN-dependant IL-15 and IL-15Rα upregulation in response to RV infection. IHC staining indicated that IL-15 was predominantly expressed on the apical surface of epithelial cells lining the airway as well as infiltrating leukocytes in the subepithelium. The location and morphology of these cells was consistent with them being activated DCs that are part of the complex network that lie directly beneath the airway epithelial layer.[33] McGill *et al.*[17] demonstrated higher levels of surface IL-15 on pulmonary plasmacytoid (pDCs) and CD8α^{+} DCs compared with alveolar macrophages after influenza infection, and IL-15 trans-presentation by these DC subsets promoted CD8^{+} T-cell survival and accumulation in the lungs.

We demonstrate that, even in the absence of type I IFN signaling, previously reported to be critical for activation of antiviral NK cell and CD8^{+} T-cell responses, IL-15c was able to rescue these responses and even boost them above levels observed in wt mice. Thus, we have identified IL-15 (and IL-15Rα) as the only ISGs necessary for these key antiviral lymphocyte responses. One other study has demonstrated the ability of exogenous IL-15 to mediate the accumulation of proliferating NK cells independently of type I IFNs;[22] however, the effect on activation of antiviral lymphocyte subsets and IFN-γ expression during virus infection was not investigated. We observed rapid and enhanced induction of lung *Ifnγ* mRNA followed by CXCL9 protein expression, which preceded the increased recruitment of IFN-γ-expressing NK and T cells with IL-15c treatment. These data identify the capacity of IL-15 to mediate *Ifnγ* expression and lymphocyte responses independently of type I IFN signaling *in vivo*.

A key question is how does IL-15c restore NK- and Th1-cell responses during RV infection in the absence of ISG expression? Especially since many lymphocyte-recruiting chemokines such as CXCL10 are induced by type I IFN signaling, which are deficient in RV-infected IFNAR1$^{-/-}$ mice.[10] This raises the possibility that the IL-15c-augmented early IFN-γ expression induced production of nontype I IFN-dependant chemokines, which could recruit NK and T cells to the lung. CXCL9, CXCL10, and CXCL11 all bind CXCR3 on NK cells and CD4^{+} and CD8^{+} T cells to mediate chemotaxis to sites of inflammation. Although originally described as interferon gamma-inducible protein 10 (IP-10), IL-15c treatment failed to induce CXCL10 production in IFNAR1$^{-/-}$ mice (despite robust *Ifnγ* expression), whereas RV-induced expression was further boosted in wt mice, suggesting that CXCL10 is dependent on type I rather than type II IFNs. In contrast, we observed robust expression of CXCL9 (originally identified as monokine induced by gamma interferon) in both wt and IFNAR1$^{-/-}$ mice peaking at 24 h. The requirement of IFN-γ for CXCL9 expression has been observed during *Klebsiella* infection using IFN-γ knockout mice.[34] Pertaining to lung responses, treatment of cultured human bronchial epithelial cells with IFN-γ induced CXCL9 expression.[35] We suggest that the early induction of *Ifnγ* gene expression in the lung, potentially stimulated by the combination of RV infection/TLR activation signals in conjunction with enhanced IL-15c stimulation of resident DCs and macrophages, was critical to recruitment of IFN-γ-expressing NK cells and T cells via enhanced expression of CXCL9. We have previously demonstrated that human macrophages infected with RV *in vitro* produce IFN-γ.[30] This is the first report to our knowledge demonstrating RV infection *in vivo* induces expression of CXCL9 in the airways, which is potentially the critical CXCR3-binding chemokine necessary for recruitment of NK cells and CD8^{+} T cells to lungs/airway. IL-15 is also reported to have direct chemotactic effects on NK cells and CD8^{+} T cells as demonstrated by migration through transwell systems to IL-15 supplemented media.[36,37] The increased numbers of NK cells observed following exogenously administered IL-15 may have also been a result of proliferation within the lung as IL-15 has been reported to stimulate NK-cell proliferation during cytomegalovirus and influenza infection.[22,37]

IL-15c augmented expression of ISGs (OAS1, PKR, and CXCL10) in wt mice during RV infection, which we suspect was through increased signaling from the type I IFN receptor. However, while *Ifnβ* mRNA was increased, BAL IFN-β protein levels were not, and BAL IFN-α protein was actually lower in the IL-15c-treated group. This was potentially due to increased type I IFN receptor expression with IL-15c treatment, resulting in increased ligand binding and less free detectable IFN in the BAL. This hypothesis is supported by the increased BAL type I IFN proteins observed in IL-15c/RV-treated IFNAR1$^{-/-}$ mice. One study has reported the capacity of IL-15 to induce IFN-β expression in macrophages *in vitro*,[38] whereas another report described IL-15-induced type I IFN by cultured human DCs.[39] IL-15c treatment also augmented type III IFN production independently of type I IFN signaling. This study is the first to demonstrate that IL-15 treatment can augment IFN and ISG expression during a viral infection *in vivo*. Although IL-15c treatment could boost ISG expression through increased type I IFN signaling, the increased endogenous *Il15* and *IL15rα* expression detected also occurred in the absence of type I IFN signaling, suggesting that IL-15 was capable of increasing its own expression and signaling independently of type I IFN. Boosted expression of IL-15 and its receptor, in conjunction with endosomal recycling of membrane-bound IL-15–IL-15Rα complexes, which allows longer and persistent IL-15 signalling,[40,41] could contribute to the enhanced lymphocyte responses and cytokine and chemokine expression detected in IL-15c-treated mice.

Our data provide novel insight into the immune response to RV infection providing first *in vivo* evidence of the importance of IL-15 for NK-cell, CD8$^+$ T-cell, and CD4+ T-cell responses to RV infection. In addition, we have identified a novel function for IL-15 in augmenting antiviral IFN-γ expression and lymphocyte responses independently of type I IFNs. Our data suggest that boosting NK and CD8+ T-cell responses during RV infection in healthy subjects is unlikely to provide substantial benefit, as the virus is efficiently cleared without causing disease. In the case of asthma exacerbations, RV infection can augment lung inflammation driven by type-2 immunity. In this case, we would predict that increasing IFN-γ production by recruitment of IL-15-activated NK and CD8+ T cells may inhibit production of type-2 cytokines and associated immunopathological responses such as airway hyper-reactivity and mucus secretion. Asthmatic bronchial epithelial cells also exhibit deficient antiviral responses and are more permissive for viral replication.[27] Boosting antiviral cellular responses may also compensate for this asthma-associated immune defect. Our data support the view that therapeutic administration of IL-15c during asthma exacerbations would be the most beneficial approach with the important caveat that therapeutic administration of IL-15c has the potential to induce exaggerated type-1 immune responses with excessive IFN-γ production and associated airways inflammation.[42] Thus, IL-15c must be developed and used with caution. This could be achieved by detailed titration studies to identify a dose range that stimulates type-1 immunity that is sufficient for better control of viral infections and suppression of type-2-driven inflammation and results in a net reduction in airways inflammation and disease.

METHODS

Human experimental rhinovirus infection and measurement of nasal cytokines. Two cohorts of 11 healthy, nonsmoking volunteers with no history of asthma or respiratory disease, negative skin prick tests, no evidence of bronchial hyper-reactivity ($PC_{20} > 8\,mg\,ml^{-1}$) and no cold in the last 6 weeks were entered in this study. Infection was induced with rhinovirus serotype 16 via nasal spray into both nostrils.[30] For one cohort, nasal responses were measured. Before RV infection (baseline) and at days 2, 3, 4, 5, 7, and 10 post inoculation, the Nasosorption technique[43–45] was used to sample soluble mediators in the nasal mucosa. IL-15 was measured using the Meso-Scale Discovery (MSD) platform (sensitivity of $< 0.6\,pg\,ml^{-1}$). The second cohort has been described previously.[30] Bronchial biopsies were obtained at baseline (2 weeks before infection) and at day 4 after infection.

Mouse rhinovirus infection. IL-15Rα$^{-/-}$ mice on a B6.129 background, B6.129 control mice (both purchased from the Jackson Laboratory, Bar Harbor, ME), and IFNAR1$^{-/-}$ mice on a C57BL/6 background were bred in house under specific pathogen-free conditions. Balb/c and C57BL/6 control mice were purchased from Harlan (Harlan–Sprague–Dawley, Shardlow, UK). Rhinovirus sertotype 1B (RV-1B) obtained from the American Type Culture Collection (Salisbury, UK) was grown in HeLa cells (European Collection of Cell Cultures) and purified for *in vivo* use as previously described.[9] The virus was inactivated by exposure to UV light at $1{,}200\,mJ\,cm^{-2}$ for 30 min. Mice were lightly anesthetized with isofluorane and infected intra-nasally (i.n.) with 50 µl of RV-1B (5×10^6 $TCID_{50}$) or PBS (mock-infected represented as 0 h in time course studies). All mouse experiments were performed using 6- to 8-week-old mice, and only female mice were used in studies involving wt Balb/c, wt B6.129, and IL-15Rα$^{-/-}$ mice, and males and females were used in studies involving wt C57BL/6 and IFNAR1$^{-/-}$ mice.

Anti-IL-15 antibody and IL-15 complex treatment. Balb/c mice were treated i.n. with 20 µg of an IL-15-neutralizing antibody (M96, Amgen, Thousand Oaks, CA) or mouse IgG2a isotype control (R&D Systems, Abingdon, UK) at the time of infection (0 h) and 24 h after infection. In other studies, IFNAR1$^{-/-}$ and C56BL/6 control mice were treated i.n. with 1.5 µg IL-15 complexed to IL-15Rα (IL-15c) (eBiosciences, Hatfield, UK) at the time of infection.

Cytokine ELISA. For BAL fluid, ELISAs for mouse IFN-α, IFN-β, IFN-λ2/3 (IL-28A/B), CXCL9, and CXCL10 (R&D Systems) were used. The right azygous, cardiac and diaphramic lung lobes were excised post BAL and homogenized in PBS with EDTA-free protease cocktail inhibitor (Roche, Indianapolis, IN); the clarified supernatant after centrifugation was used for IL-15 (R&D Systems) and IL-15c (eBiosciences) ELISAs according to the manufacturer's recommended protocol.

TaqMan quantitative real-time PCR. The mouse apical lung lobe was excised and stored in RNA later (Qiagen, Manchester, UK) before total RNA extraction (RNeasy miniprep kit, Qiagen), and 5 µg of RNA was reverse-transcribed for cDNA synthesis using random hexamers as primers (Omniscript RT kit, Qiagen). RNA from BAL cells was also extracted and converted to cDNA by the same process. Quantitative PCR was conducted using primers and probes specific to each gene analyzed, using an ABI 7500 TaqMan (ABI Foster City, CA). Each gene was normalized to 18 s rRNA and expressed as mRNA copies per microliter of cDNA reaction, quantified by a standard curve from amplified plasmid DNA standards.

Immunohistochemistry. Human bronchial biopsies were embedded in Tissue Tek II OCT, frozen within 15 min in isopentane pre-cooled in

liquid nitrogen, and stored at −80 °C. The best frozen sample was then oriented and 6μm thick cryostat sections were cut for immunohistochemical light microscopy analysis. After blocking nonspecific binding, 1.3 μg ml^{-1} mouse anti-human IL-15 (Santa Cruz Biotechnology sc-73311, Dallas, TX) were applied for 1 h at room temperature in a humidified chamber. As positive control, we have used human nasal polyps and these were used for negative control slides, which were treated with normal mouse nonspecific immunoglobulins (Santa Cruz Biotechnology). After repeated washing steps with TBS, the sections were subsequently incubated with a horse anti-mouse biotinylated antibody (Vectastain Elite ABC Kit, Vector Laboratories, Peterborough, UK) for 30 min at room temperature. After further washing, the sections were subsequently incubated with ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories) for 30 min at room temperature. Slides were then incubated with chromogen-fast diaminobenzidine (DAB) as a chromogenic substance, after which they were counterstained in haematoxylin and mounted on aqueous mounting medium.

Single IHC staining of mouse lung sections from formalin-fixed paraffin-embedded mouse lungs was performed as previously described.[10] After blocking slides (5% rabbit serum), goat anti mouse IL-15 (RnD Systems AF447) was applied at 4 μg ml^{-1}. For the negative control slides, normal goat nonspecific immunoglobulins (Santa Cruz Biotechnology) were used. The intensity and distribution of positive staining in human biopsies and mouse lung sections was evaluated using a standard four point scale, with sections being scored as 0, 1, 2, or 3 according to a validated method.[46]

Flow cytometry. The left lung lobe was excised post BAL, crudely dissociated using the GentleMACS tissue dissociator (Miltenyi Biotech, Surrey, UK) and digested upon incubation at 37 °C in buffer containing 1 mg ml^{-1} collagenase Type XI and 80 units ml^{-1} Bovine Pancreatic DNase Type IV (both Sigma-Aldrich, Dorset, UK). For intracellular cytokine staining (ICS), cells were stimulated with PMA (50 ng ml^{-1}) and ionomycin (500 ng ml^{-1}) using BD GolgiStop (BD Biosciences, Oxford, UK) for 3 h at 37 °C. Lung and BAL cells were incubated with anti-mouse CD16/CD32 (FC Block, BD Biosciences) before staining for cell surface markers: CD3e (clone 500A2), CD4 (clone RM4-5), CD8a (clone 53-6.7), NK1.1 (clone PK136), NKp46 (clone 29A1.4), and CD69 (clone H1.2F3) (all BD Biosciences). Cells were washed and stained with Live/Dead fixable dead cell stain kit (Invitrogen, Paisley, UK), followed by incubation with BD Fix/Perm solution (BD Biosciences). For ICS, cells were stained for IFN-γ (clone XMG1.2, BD Biosciences) and granzyme B (clone GB12, Invitrogen) in BD PermWash^{TM} (BD Biosciences). Data were acquired using a BD LSR II digital flow cytometer (BD Biosciences) and BD FACS Diva software (Oxford, UK). Analysis was performed using FlowJo 9.3.1.2 software (Oxford, UK).

Statistical analyses. For human studies, IL-15 data are presented as mean (± s.e.m.). Baseline and infection peak data were comparatively analyzed with the Mann–Whitney test. Correlations between data sets were examined using Spearman's rank correlation coefficient for nonparametric data. Differences were considered significant for all statistical tests at $P<0.05$. All reported P-values are two sided. Animal experiments involved 4–7 mice per group in up to three independent experiments. Data were analyzed using two-way ANOVA and Bonferroni's multiple comparison test. All statistics were calculated using Prism 4 software (Graphpad, La Jolla, CA), with $P<0.05$ taken as significant.

Study approval. The human infection studies were approved by St Mary's National Health Service Trust Research Ethics committee. All subjects gave written informed consent. All animal work was completed in accordance with UK Home Office guidelines following approval via the ethical approval process (UK project licence PPL 70/7234).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declare no conflict of interest.

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