

ORIGINAL ARTICLE

IFN β inhibits the development of allergen tolerance and is conducive to the development of asthma on subsequent allergen exposure

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Abstract

Asthma is a chronic disease affecting up to 10% of the Australian population for which medical treatment is solely aimed at relief of symptoms rather than prevention of disease. Evidence from animal and human studies demonstrates a strong link between viral respiratory infections, atopy and the development of asthma. Type I IFNs include IFN α and IFN β , with subtype expression tailored toward the specific viral infection. We hypothesized that exposure to type I IFNs and allergen may interfere with the healthy response to innocuous airway antigen exposure. In this study, we use an ovalbumin (OVA)-induced BALB/c model of experimental allergic airways disease, where pre-exposure of the airways to OVA is protective against allergen sensitization, leading to allergen tolerance. We investigated airways pre-exposure with OVA and type I IFNs on development of allergic airways disease. We demonstrate restoration of allergic airways disease on pre-exposure with allergen and IFN β , and not IFN α . Dysfunction in tolerance led to changes in dendritic cell antigen capture/traffic, T-cell and B-cell responses. Furthermore, exposure to IFN β with ongoing allergen exposure led to the development of hallmark asthma features, including OVA-specific IgE and airways eosinophilia. Data indicate a role for IFN β in linking viral infection and allergy.

INTRODUCTION

Initial development of atopic asthma is strongly linked to respiratory viral infection during infancy.^{1,2} Long-term cohort studies have established that children most likely to develop persistent asthma are those who experience repeated/intense lower respiratory infections (LRIs) during the first 1–2 years of life.^{3,4} The release of type I IFN is stimulated early in the innate immune response to viral infection, is tailored to the inducing microbial agent, and is a key driver in the anti-viral response.⁵

In healthy airways the normal adaptive immune response to allergen early in life is regulated by dendritic cell (DC) antigen capture and presentation for the generation of immunologic tolerance. Failure of DC mechanisms responsible for the induction of peripheral

tolerance to usually innocuous antigens/allergens may initiate Th2 memory cell responses, which is the response underlying allergic asthma.⁴ Mechanisms controlling the development of tolerance or inflammatory response to allergen are not well understood.

The hallmark symptoms of atopic asthma in response to allergen include airway hyper-responsiveness with concomitant cellular infiltration of eosinophils, B cells, Th2 effector cells and DCs into the airways.⁴ In addition, serum levels of IgE and IgG1 antibodies are elevated and there is increased production of the cytokines IL-4, IL-5 and IL-13.⁶ During viral infection the pulmonary epithelium provides a barrier between the external environment and the internal tissues and produces type I IFN, predominantly IFN β , upon activation of viral TLRs.⁷ Of note, viral infections targeting the pulmonary

epithelium are risk factors for the development of asthma. In particular, respiratory syncytial virus (RSV) and rhinovirus (RhV) infections are associated with asthma development, can infect airway epithelial cells (AECs), and induce type I IFN production.⁷

Murine models of asthma have shown that passive aerosol delivery of innocuous antigen such as ovalbumin (OVA) normally promotes development of protective immunological tolerance.⁸ This tolerance was preceded by low-level transient IgE responses, and re-challenge of OVA-exposed mice, via intraperitoneal OVA-sensitization, resulted in significantly lower levels of IgE production and the induction of Treg cells.⁹ Other studies have indicated that early life viral infection could subvert this tolerance process and lead to allergic sensitization in animal model studies.¹⁰ In the OVA-allergen mouse model of asthma, OVA exposure during the acute phase of influenza infection inhibited tolerance induction, and infected mice developed high titers of OVA-specific IgE in response to subsequent challenge with allergen via peritoneal sensitization.¹¹ The specific immune mechanisms underlying these observations are not well understood.

The type I IFNs consist of IFN- α (13 subtypes) and a single IFN- β , in humans and mice,¹² that bind a common IFN α receptor (IFNAR). While the interaction with IFNAR relies on a number of conserved anchor points across the IFNs there are also specific interactions between IFNAR and each IFN that regulate the duration and magnitude of the signaling response. This leads to differential activation of gene expression and the observed specific activities of the IFNs.^{5,12,13} The impact of type I IFN subtypes, IFN α and IFN β , on the allergen tolerization process in healthy airways is unknown. The development of allergic asthma involves a complex series of interactions between the immune system and the environment, and there is increasing evidence that associates respiratory viral infections with the development of asthma.¹⁴ The molecular mechanisms that underpin this link remain unclear. However, experimental evidence suggests that viral infection disrupts airway mucosal tolerance mechanisms and enhances proallergic immune responses.¹⁵ Given the central role type I IFN plays in the response to viral infection, it is possible that aberrant or excessive type I IFN expression in response to respiratory viral infection may skew the normal tolerant response to allergen. We hypothesized that the perturbation of tolerance by viral infections could be mediated by the production of type I IFNs. Here, we report results of our investigations that used a murine model to test a role for the type I IFNs, IFN α and IFN β , in exacerbating the development of allergic airways inflammation to the allergen, OVA.

RESULTS

The induction of tolerance to OVA is inhibited by IFN β

Pre-exposure of the airways to OVA leads to the development of allergen tolerance upon subsequent challenge,^{8,16-18} and viral exposure in early life is linked to asthma onset.² In order to determine whether type I IFN production during viral infection interferes with allergen tolerance, we exposed mice to OVA and IFN α or OVA IFN β . In brief, mice were challenged with type I IFN intranasally (i.n) and 24 h later with allergen for three exposures, followed by sensitization with OVA/Alum and OVA challenge 10 days later, according to the schedule in Figure 1a. As previously published,⁸ OVA-sensitization of the sal/sal pretreatment group followed by OVA-challenge significantly increased eosinophil cell numbers in bronchoalveolar lavage fluid (BALF) and OVA-specific IgE in serum, compared to the OVA-sensitized, and saline-challenged control (sal/sal with sal challenge) (Figure 1b, c). Pre-exposure to OVA (sal/OVA) or IFN α with OVA (IFN α /OVA) significantly reduced airways eosinophilia and serum OVA-specific IgE levels in mice sensitized and challenged with OVA, to induce allergic airway disease. However, pre-exposure to IFN β /OVA with subsequent OVA-sensitization and OVA-challenge significantly increased airways eosinophilia compared to OVA (sal/OVA) or IFN α with OVA intranasal (IFN α /OVA) pretreatment prior to induction of allergic airway disease (with OVA). These data indicate that OVA-induced tolerance prior to allergen sensitization is abrogated by the presence of IFN β but not IFN α .

Type I IFN and allergen immune response in the airways and airway draining lymph node (ADLN)

Type I IFN is produced by the epithelium, plasmacytoid DC (pDC) and conventional DC (cDC). Type I IFNs influence both innate and adaptive immune responses via promotion of cDC differentiation,¹⁹ as well as activation and cytokine production⁵ during viral infection. We hypothesized that type I IFN administration in conjunction with allergen would impact on the recruitment of DC and OVA-uptake in the airways (Figure 2), and DC trafficking to the ADLN (Figure 3). Therefore a single saline, IFN α , or IFN β dose was administered per nasally, followed by intranasal saline or OVA 24 h later, with DC phenotypes determined 2 or 24 h later.

In the airways, CD11b^{hi} DC function in immune surveillance in naïve mice trafficking to the ADLN, whilst CD11b^{hi} and CD11b^{lo} DC traffic to the ADLN bearing allergen in mice with allergic airways disease.¹⁹ The flow cytometry gating strategy used to select for airway DC is

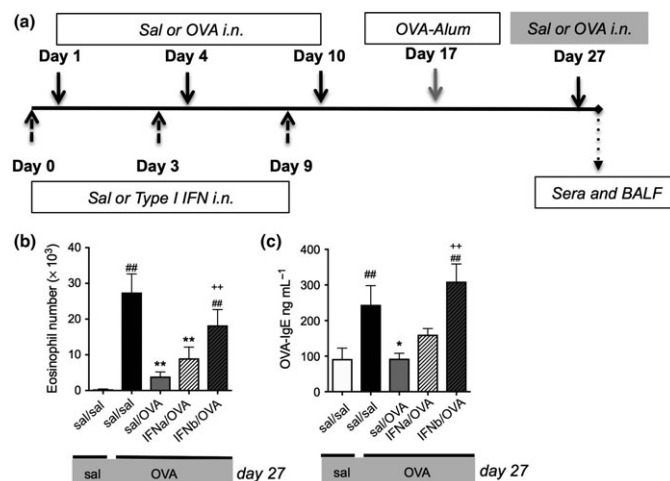


Figure 1. IFN β and allergen exposure inhibits the development of OVA-induced tolerance in asthmatic mice. Mice received 1000IU IFN α , 1000IU IFN β , or saline intranasally on day 0, 3 and 9; 24 h later (day 1, 4 and 10) mice received intranasal saline or OVA (50 μ g) as indicated. Mice were OVA-Alum sensitized (20 μ g OVA-Alum in 200 μ l) at day 17, and re-challenged with intranasal OVA or saline at 10 days postsensitization, with BALF and sera collected 24 h after the final OVA administration. **(a)** Illustration of the OVA-tolerance protocol, type I IFN administration, OVA-sensitization and OVA-challenge. **(b)** Airways eosinophil cell number. **(c)** serum OVA-specific IgE. ($n = 10$ mice/group, data representative of two separate experiments; one-way ANOVA, * $P < 0.05$. ** $P < 0.005$, compared to saline pretreatment, OVA-challenge. ## $P < 0.005$, compared to saline pretreatment, saline-challenge; ++ $P < 0.005$, compared to OVA pretreatment, OVA-challenge.)

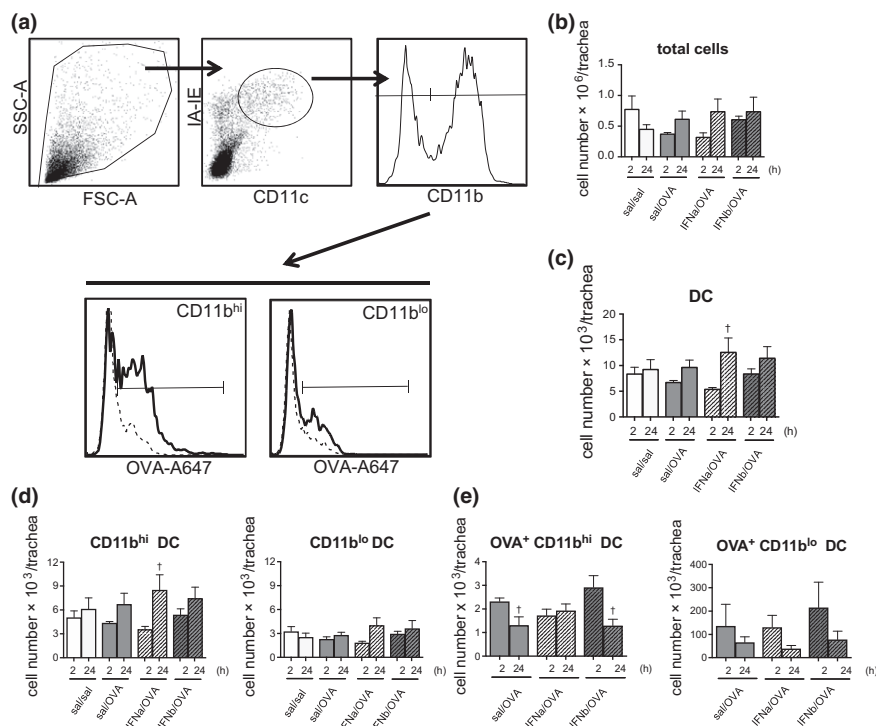


Figure 2. Dendritic-cell subpopulation number and OVA uptake in airways. Mice received 1000IU IFN α , 1000IU IFN β , or saline intranasally, with OVA-A647 challenge (50 μ g intranasal) at 24 h, and trachea harvest 2 or 24 h later. Tracheas were digested to form a single-cell suspension, and antibody stained for flow cytometry. **(a)** DC were gated as CD11c⁺MHCII⁺ cells then as CD11b^{hi} and CD11b^{lo} populations, followed by gating on OVA-A647⁺ cells. **(b)** Total cell numbers in trachea. **(c)** Total DC numbers. **(d)** CD11b^{hi} and CD11b^{lo} DC subpopulation numbers. **(e)** OVA⁺ CD11b^{hi} and CD11b^{lo} DC numbers. ($n = 6-8$ mice/group, data represent three separate experiments; unpaired t -test, $^{\dagger}P < 0.05$, compared same group 2 h time point).

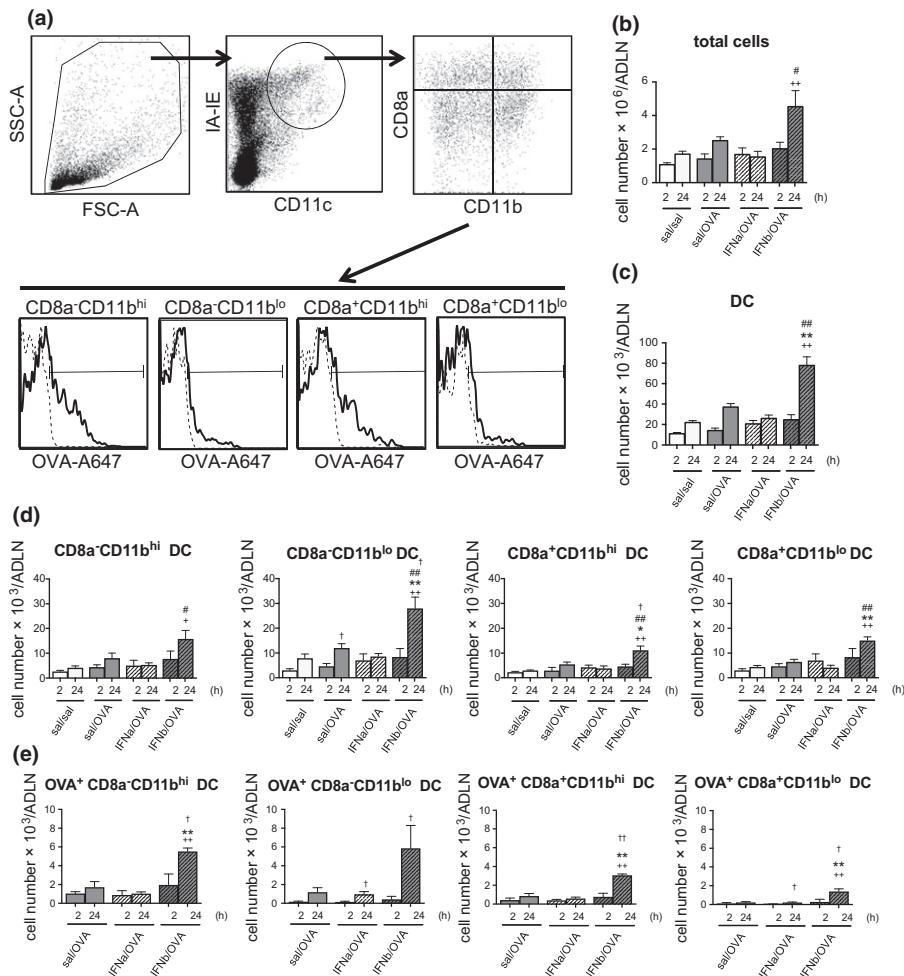


Figure 3. Dendritic cell subpopulation number and OVA delivery in ADLN. Mice received 1000IU IFN α , 1000IU IFN β , or saline intranasally, with OVA-A647 challenge (50 μ g intranasal) at 24 h, and ADLN harvested at 2 h or 24 h post final OVA-challenge. ADLN were digested to form a single-cell suspension, and antibody stained for flow cytometry. **(a)** Gating strategy, DC were gated as CD11c⁺MHCII⁺ cells, with subpopulations determined by CD8a and CD11b expression, and OVA-A647 uptake shown in histogram plots. **(b)** Total cell numbers in ADLN. **(c)** Total DC numbers. **(d)** Cell numbers for DC subpopulations CD8a⁻CD11b^{hi}, CD8a⁻CD11b^{lo}, and CD8a⁺CD11b^{hi} and CD8a⁺CD11b^{lo}. **(e)** OVA⁺ DC numbers in ADLN subpopulations. ($n = 3$ or 4 mice/group, data represent four separate experiments. One-way ANOVA, $*P < 0.05$. $**P < 0.005$, compared to saline-OVA respective time point; $\#P < 0.05$. $##P < 0.005$, compared to saline-saline respective time point; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.005$ compared to IFN α /OVA respective time point; $^{\ddagger}P < 0.05$. Two-tailed, unpaired t -test, $^{\dagger\dagger}P < 0.005$ same group 2 and 24 h time point).

shown in Figure 2a. The total number of tracheal cells was not significantly changed between groups exposed to saline/saline, saline/OVA, IFN α /OVA or IFN β /OVA at the 2- or 24-h time point (Figure 2b). There was a significant increase in the numbers of DC observed in mice of the IFN α /OVA treatment between 2- and 24-h (Figure 2c), with no other significant changes in total DC number between the 2- or 24-h time points in other treatment groups. The change in DC numbers between the 2- and 24-h time points for IFN α /OVA was largely driven by an increase in CD11b^{hi} DCs (and less so CD11b^{lo} DCs) in the trachea (Figure 2d). Next we examined OVA capture in airway DC subset as

determined by OVA-A647 uptake. Of note, OVA⁺ CD11b^{hi} DC significantly declined with sal/OVA and IFN β /OVA between 2 and 24 h, potentially indicating egress of OVA⁺ DC from the airways to the ADLN (Figure 2e).

Next we examined the DCs in the ADLN, with the flow cytometry gating strategy depicted in Figure 3a. Notably there were significantly elevated numbers of ADLN cells (Figure 3b), with a substantial increase in the numbers of DCs 24 h after intranasal OVA in the IFN β /OVA treatment group (Figure 3c). Airways trafficking DC subpopulations, CD8a⁻CD11b^{hi} and CD8a⁻CD11b^{lo}, and resident CD8a⁺CD11b^{hi} and CD8a⁺

CD11b^{lo} DCs, were increased in the ADLN of mice of the IFN β /OVA treatment group (Figure 3d), of which many harbored OVA antigen (Figure 3e). In contrast, IFN α /OVA treatment had minimal impact on subpopulation numbers or OVA⁺ DC numbers in the ADLN (Figure 3d, f). These data suggest an increased number of OVA⁺ DC in the ADLN that may contribute to subsequent OVA-induced inflammation and allergy.

To determine the effects on T-cell responses, we harvested ADLN at 4 days post sal/sal, sal/OVA, IFN α /OVA and IFN β /OVA challenge (Figure 4). ADLN total cell numbers at day 4 post IFN and/or OVA challenge were not significantly different from the saline/saline control (saline/saline, $1.68 \pm 0.19 \times 10^6$ cells/ADLN; saline/OVA, $3.22 \pm 0.36 \times 10^6$ cells/ADLN; IFN α /OVA, $1.89 \pm 0.16 \times 10^6$ cells/ADLN; and IFN β /OVA $3.29 \pm 0.57 \times 10^6$ cells/ADLN; mean $\pm$ s.e.). Significantly increased numbers of CD4 T cells in the ADLN were observed 4 days post OVA-challenge for mice from the sal/OVA and IFN β /OVA treatments, compared to mice of the sal/sal treatment (Figure 4a). Most notably, there were signs of increased CD4 T-cell proliferation, as determined by upregulation of Ki67, in mice from the IFN β /OVA treatment (Figure 4b), indicative of an activated CD4 T-cell response.

Finally, to determine a Th1/Th2 immune response in bronchoalveolar lavage fluid we determined cytokine levels at 24 h sal/OVA, IFN α /OVA and IFN β /OVA by bioplex assay (BioRad, Gladesville, NSW, Australia; and one-way ANOVA, Benjamin and Hochberg, FDR = 0.1). A significant increase in IL-4 was determined for IFN α /OVA and IFN β /OVA compared to sal/OVA (26.8 ± 6.9 pg mL⁻¹, 190.2 ± 13.9 pg mL⁻¹, and 318.49 ± 103.3 pg mL⁻¹, respectively). IL-12p40 was significantly

upregulated in IFN α /OVA (519.1 ± 59.8 pg mL⁻¹) and IFN β /OVA (673.6 ± 73.6 pg mL⁻¹) compared to sal/OVA (283.2 ± 25.5 pg mL⁻¹), and IFN β /OVA IL-12p40 levels were significantly increase compared to IFN α /OVA. In addition, IL-17 was significantly increased in IFN β /OVA (26.5 ± 4.5 pg mL⁻¹) compared to sal/OVA (16.7 ± 2.1 pg mL⁻¹) and IFN α /OVA (15.33 ± 2.7 pg mL⁻¹). These data indicate, after OVA and type I IFN induction of IL-4, a hallmark feature of allergic asthma. In addition, we observed IL-17 production after IFN β and allergen exposure. Interestingly, other studies in mice tolerant to allergen have shown asthma-linked symptoms on RSV infection that were IL-17 dependent.²⁰

Repeated IFN β /OVA maintains a T-cell response, induces T follicular helper (Tfh) cell responses, stimulates low-level IgE production and airways eosinophilia

Next, we determined CD4 and CD8 T-cell responses in the ADLN after repeated exposure to allergen in conjunction with type I IFN, using our tolerizing protocol, with ADLN harvested at day 11 (Figure 1a). Significantly elevated CD4 and CD8 T-cell numbers were observed in mice from the IFN β /OVA exposure treatment compared to saline/OVA exposure alone (Supplementary figure 1). In addition, there was a sign of increased CD8 T-cell proliferation in the ADLN, as determined by Ki67 expression, in mice from IFN β /OVA treatment compared to those from the saline/saline control and saline/OVA treatments. No significant change in CD4 CD25⁺, CD8 CD25⁺ or CD4 Treg cells was observed between groups. These data indicate changes in CD4 and CD8 T-cell number and proliferation after repeated exposure to IFN β /OVA after 11 days (Figure 1a).

Next, we examined ADLN for germinal center formation (see Figure 5a). Induction of T follicular helper (Tfh) cells is central to germinal center formation and production of IgG antibodies that precedes class switch recombination to IgE in the presence of IL-4 in secondary lymph nodes. Therefore, we measured the expression of CD4 cell surface expression markers CD154 (CD40L) and CD185 (CXCR5) that are involved in recruitment of Tfh to germinal centers. Notably, we determined a significant increase in CD154 expression on CD4 T cells from the ADLN after repeated exposure to IFN β and OVA, compared to the sal/OVA treated group (Figure 5a). No significant increase was observed in the IFN α group, although there was a similar trend to that observed for IFN β (Figure 5b). A trend for increased CD185 expression on CD4 T cells was observed after IFN α /OVA and IFN β /OVA exposure, suggestive of increased Tfh cells. These data suggest the effects of both

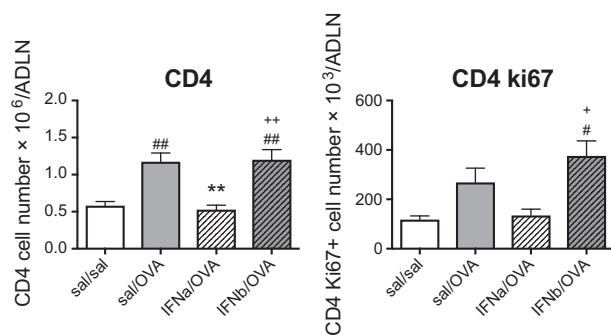


Figure 4. T-cell responses after IFN and OVA delivery in ADLN. Mice received 1000IU IFN α , 1000IU IFN β , or saline intranasally, with OVA-challenge (50 μ g intranasal) 24 h later, with ADLN harvested 4 days later. ADLN were digested to single-cell suspension, antibody stained for flow cytometry and T cells gated on CD4 and Ki67. (a) Total CD4 cell numbers. (b) CD4 Ki67 cell numbers. ($n = 3-6$ mice/group; one-way ANOVA. ** $P < 0.005$, compared to saline- OVA challenge; # $P < 0.05$. ## $P < 0.005$, compared to saline-saline challenge; * $P < 0.05$, ** $P < 0.005$ compared to IFN α /OVA challenge.

IFN α and IFN β are similar on the T and T_{fh} cell populations in this model.

There was a trend for increased OVA-specific IgE levels in serum after 11 days in mice for the intranasal sal/OVA treatment, compared to levels observed in mice of the sal/sal treatment as measured via ELISA. Furthermore, testing of serum by passive cutaneous anaphylaxis determined a significant increase in the reciprocal titer of sal/OVA (1.60 ± 0.35 ; $P < 0.001$) compared to IFN α /OVA and IFN β /OVA (data not shown). In order to boost OVA-IgE levels in sera we challenged mice again with OVA at 7 days post the final OVA exposure in the pretreatment protocol (Figure 1a). On further OVA-challenge of mice at day 17 there was significantly elevated OVA-specific IgE in serum after IFN β /OVA exposure (Figure 5b). These data indicate that with repeated OVA challenge OVA-IgE levels in serum increase after IFN β /OVA pretreatment.

Finally, as OVA-specific IgE is linked with increased airways inflammation, we determined airways eosinophilia 11 days after the intranasal exposure began, or with the additional OVA-challenge at day 17 in BALF at 24 h post

final OVA-exposure. No increased airways eosinophilia was observed at day 11 or day 17 (data not shown). We therefore further exposed mice to OVA on days 17, 21 and 28 (as per Figure 5a) with harvest 24 h after the final OVA administration in order to drive eosinophil airways inflammation. Eosinophilic airways inflammation was significantly increased compared to sal/sal, sal/OVA or IFN α /OVA-exposed mice (Figure 5d). This indicates development of an asthmatic response in mice treated with IFN β /OVA and repeatedly challenged with allergen, indicative of the induction of an allergic response in the airways.

Early IFN β /OVA exposure followed by repeated allergen exposure induces T- and B-cell changes in the ADLN

IFN α and IFN β reportedly impact on T- and B-cell responses and therefore we sought to determine whether there were significant changes to immune cell activation and phenotype after IFN and allergen exposure. We first examined CD4 and CD8 T-cell responses in the ADLN

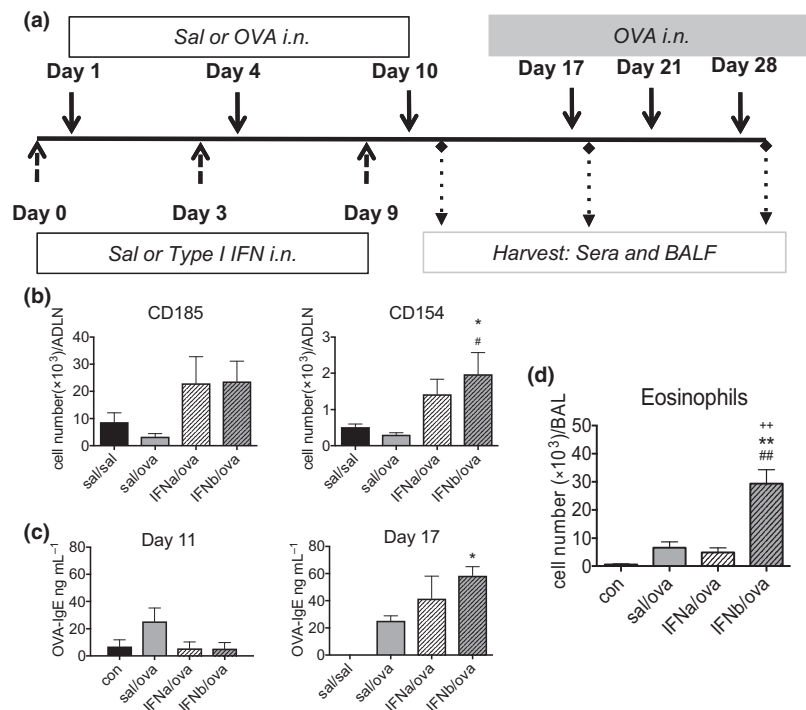


Figure 5. Airways IFN β and repeated allergen challenge induces germinal center formation, increased serum OVA-IgE and eosinophilic airways inflammation. **(a)** Schematic demonstrating timing of type I IFN and OVA administration and tissue harvest. Mice received intranasal instillation of saline, 1000IU IFN α , 1000IU IFN β , or OVA (50 μ g) at the indicated time points. Tissues were collected at 24 h after the nominated final OVA administration and digested to single-cell suspension, and stained for flow cytometry. Sera were prepared from whole blood and BALF was collected. **(b)** T-cell numbers expressing CD185 and CD154 after day 11 OVA administration. **(c)** OVA-specific IgE in sera at after day 11 or day 17 OVA administration (n.d., not done). **(d)** Airways BALF eosinophilia after repeated OVA challenge to day 28. ($n = 5$ mice/group, data representative of two separate experiments. One-way ANOVA, * $P < 0.05$. ** $P < 0.005$, compared to OVA pretreatment, OVA challenge; # $P < 0.05$. ## $P < 0.005$, compared to saline pretreatment, OVA-challenge; * $P < 0.05$, ** $P < 0.005$ compared to IFN α /OVA pretreatment, OVA challenge).

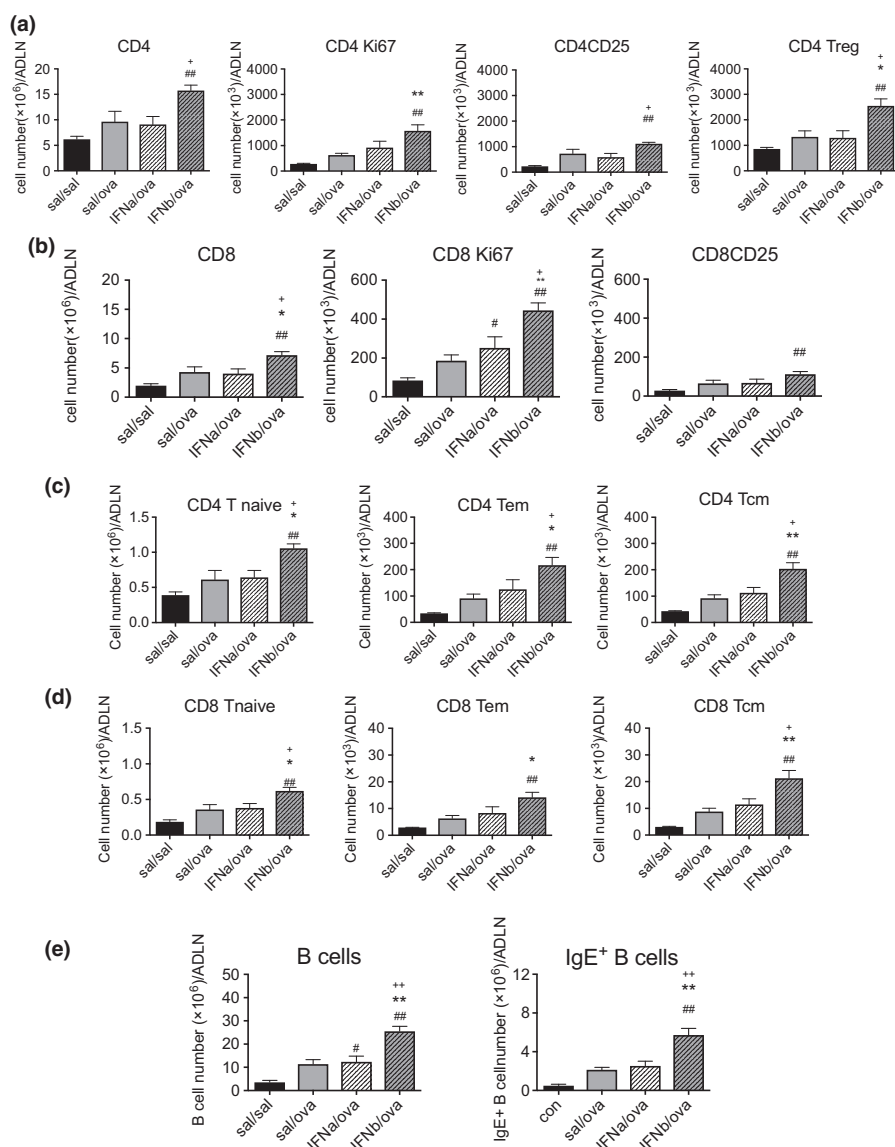


Figure 6. IFN β and repeated allergen challenge enhances T- and B-cell responses in the ADLN. Mice received 1000IU IFN α , 1000IU IFN β , or saline intranasally on day 0, 3 and 9; 24 h later mice received intranasal saline or OVA (50 μ g) as indicated. Mice were subject to further saline OVA (50 μ g) intranasally at day 17, 21 and 27 (as per Figure 5a). ADLN were collected at 24 h after the final OVA administration. ADLN were digested to single-cell suspension. **(a)** CD4, CD4 Ki67, CD4 CD25⁺ and CD4 Treg cell numbers. **(b)** CD8, CD8 Ki67 and CD8 CD25⁺ cell numbers. **(c)** CD4 T naïve, Tem and Tcm cell numbers **(d)** CD8 T naïve, Tem and Tcm cell numbers. **(e)** Total B cell and IgE⁺ B-cell numbers. (n = 5 mice/group; one-way ANOVA, * P < 0.05. ** P < 0.005, compared to OVA pretreatment, OVA challenge; # P < 0.05. ### P < 0.005, compared to saline pretreatment, OVA-challenge; * P < 0.05, ** P < 0.005 compared to IFN α /OVA pretreatment, OVA challenge).

after OVA and/or type I IFN treatment with subsequent repeated OVA exposure on days 17, 21 and 28 (Figure 5a). Increased numbers of total CD4 cells, proliferating CD4 cells (Ki67⁺), activated CD4 CD25⁺ (FOXP3⁻) cells, and CD4 Treg cells were observed after IFN β /OVA treatment (Figure 6a). Similarly, there were increased numbers of CD8 cells that were proliferating (Ki67⁺), and activated (expressed CD25) following IFN β /OVA treatment and repeated OVA-challenge (Figure 6b).

Subsequently we examined the balance of naïve (CD62L⁺ CD44⁻), effector memory (Tem; CD62L⁻ CD44⁺) and central memory (Tcm; CD62L⁺ CD44⁺) T-cell numbers in the ADLN (Figure 6c, d). There were significant increases in all of these T-cell populations (both CD4 and CD8) in mice of the IFN β /OVA treatment with subsequent OVA challenges at 17, 21 and 18 days of the protocol, compared to numbers measured in mice of other treatment groups. Finally, this was accompanied by

a significant increase in the numbers of total B cells and IgE⁺ B cells in mice from the OVA/IFN β treatment (Figure 6e).

Together, these data indicate that immune dysregulation occurs with early exposure to allergen in the presence of IFN β , and with subsequent ongoing respiratory challenges with allergen drives allergic sensitization.

DISCUSSION

Airways exposure to allergen has previously been shown to be protective against the development of experimental allergic airways disease after allergic sensitization allergen protocols,⁸ that is, mice are tolerant to OVA. Interestingly, viral infection at the time of exposure to tolerizing doses of OVA leads to mice that developed allergic airways disease in response to allergen sensitization protocols; that is, tolerance to allergen was broken by this concurrent viral infection.¹¹ As type I IFNs are released in response to viral infection we postulated that cytokine exposure at the time of administration of tolerizing OVA doses would similarly lead to mice developing allergic airways disease after allergen sensitization. In this study, we observed a dysregulation of allergen tolerance mechanisms after exposure to a combination of OVA and IFN β , but not OVA and IFN α . In particular, modulation of airway and draining lymph node dendritic cell function, T-cell response, B-cell responses, and airways inflammation are shown to occur after IFN β and allergen exposure, and allergen sensitization. Finally, we show that IFN β and OVA exposure, followed by repeated OVA exposure, led to development of the hallmark features of experimental allergic airways disease, including elevated OVA-specific IgE and airways eosinophilia.

The normal response of healthy airways to antigen exposure is the development of immune tolerance. However, tolerance development is interrupted during viral infection, potentially providing an opportunity for the later development of atopy and asthma.¹¹ Initially we determined that OVA exposure prior to OVA-sensitization was protective against onset of experimental allergic airways disease, in keeping with previous findings.⁸ However, where OVA exposure was in conjunction with IFN β , mice went on to develop experimental allergic airways disease after OVA-sensitization protocols and stimulation with allergen. Recent evidence from the Childhood Asthma Study cohort demonstrated a strong link between infant severe lower respiratory tract infections, in particular RhV and RSV, and the development of asthma.^{3,14} The initial line of defense during viral infection is the induction of type I IFNs and it is postulated here that this cytokine modulates tolerance mechanism. This study indicates an important role for IFN β in the dysregulation of allergen exposure tolerance mechanisms.

Type I IFNs link the innate and adaptive immune response, promoting DC differentiation,¹⁹ activation and cytokine production,⁵ to modulate adaptive immune responses. The plasmacytoid DC (pDC) are the most potent producers of type I IFNs, predominantly the IFN α subtypes, which subsequently modulate conventional DC (cDC) proliferation, maturation and apoptosis as well as antigen and migratory presenting capacity.²¹ The cDCs also respond directly to pathogen with production of lower levels of type I IFNs.⁵ Notably, cDCs are the primary responders to inhaled allergen, specializing in sampling the local antigenic environment of the airways. Allergen bearing cDCs which traffic from the airways to the ADLN induce T-cell tolerance or an inflammatory immune response.^{22, 23} This is dependent on the DC phenotype (cell surface marker expression), cytokine expression profile (including type I IFN and IFN stimulated gene expression), as well as the lymph node cytokine milieu.^{24,25} In the airways, CD11b⁺ DC function in immune surveillance in naïve mice, whilst CD11b⁺ and CD11b[−] DC traffic to the lymph node bearing allergen in allergic airways inflammation.^{21–24} We show IFN β increased traffic of CD8a[−] CD11b^{hi} and CD8a[−] CD11b^{lo} OVA⁺ DCs to the ADLN, and increased OVA⁺ CD8a⁺ CD11b^{hi} and CD8a⁺ CD11b^{lo} DC in the ADLN. This increased frequency of OVA⁺ DC in the ADLN potentially modulates the subsequent immune response to allergen that would normally lead to the development of allergen tolerance in the airways.

Type I IFNs have been reported to stimulate activation of T-cell responses.²⁶ In addition, type I IFN signaling on naïve CD8 T cells is critical to support their expansion, facilitate effector functions and induce heightened responses to cytokines,^{27,28} events crucial antiviral immunity. Type I IFNs have also been implicated in stimulating memory T-cell responses.^{27,28} We show increased CD4 T cell and CD8 T-cell number and/or proliferation with IFN β /OVA exposure. In addition, after IFN β /OVA exposure and repeated airways challenge with OVA allergen there remained an increased number and proliferative response in CD4 and CD8 T cells indicative of an immune response to allergen that was not seen in OVA-tolerized mice. Furthermore, continuing OVA exposure after OVA/IFN β treatment led to an elevation of T_{naive}, T_{em} and T_{cm} populations the latter indicating a memory response to allergen. These study data indicate a role for IFN β in stimulation of the T-cell responses on allergen exposure.

Type I IFNs have been reported to modulate both B cell and antibody responses.^{21, 29, 30} Data in this study indicate an increase in B-cell numbers and IgE⁺ B cells after IFN β /allergen exposure. Furthermore, serum OVA-specific IgE levels were elevated after airways IFN β /OVA exposure and repeated airway OVA challenge. In addition, in IFN β /OVA with chronic OVA exposure, we show

upregulation of T-cell surface markers CD154 (CD40L) and CD185 (CXCR5) indicative of T follicular helper cells important for migration to germinal centers and/or B-cell help. Other studies indicate type I IFN in promotion of increased B-cell survival *in vitro*,²⁹ induction of antibody class switching,³¹ and stimulation of T- and B-cell germinal center activity.^{32, 33} Interestingly, other studies have demonstrated a reduced antibody response, and isotype switching, in mice lacking IFNAR expression on B and T cells.³⁰ This study indicates a role for IFN β in germinal center formation and development of OVA-specific IgE response.

In this study, IFN β promoted airways inflammation in response to allergen exposure, strongly suggesting IFN β is critical to the development of allergic asthma. Similarly, in a murine house dust mite (HDM) model of allergic asthma sensitization in IRF3^{-/-} C57BL/6 mice, which have reduced IFN β production, specific Th2 cell responses failed to develop, with decreased airways cellular inflammation and airway hyper-responsiveness, due to impaired DC maturation and migration.³³ Studies in human peripheral blood mononuclear cells infected with rhinovirus after depletion of pDC in culture markedly inhibited IFN α secretion, and led to a significant increase in the expression of the Th2 cytokines IL-5 and IL-13.³¹ Other human studies have indicated that deficient IFN α production and excess production of IFN β may be important in the pathophysiology of asthma in smokers.³⁴ Furthermore, depletion of pDCs, which have been shown to produce high levels of IFN α ³⁵ inhibits the development of tolerance to allergens and enhances allergic airway inflammation.³⁶ It is possible therefore that the reduced IFN α expression in this model produced an IFN β dominant response disrupting the development of airways tolerance to allergen.

In summary, IFN β inhibited the development of tolerance to allergen exposure in the airways, whereas IFN α had no impact in this model. IFN β and allergen co-exposure exacerbated the development of immune dysfunction on subsequent allergen exposure that promoted the development of hallmark asthma features, airways eosinophilia and increased numbers of IgE⁺ B cells. Interestingly, when IFN α was co-administered with allergen, intranasal tolerance was largely preserved, indicating that different immune responses may be induced by specific type I IFN subtypes. It should be noted that this study used 8-week-old mice as the model which is equivalent to young adult humans, whereas the human data shows that the link between respiratory viral infection and asthma is very early in life. Whilst this model is commonly used and the maintenance of mice in pathogen free environments supports the use of this approach to model early pathogen exposure, further work to confirm the relevance to human pathology will be important. From these findings it is

plausible that dominant IFN β responses to viral infection in infancy or early childhood may predispose these individuals to later development of allergen sensitization that manifests with chronic allergen exposure. Furthermore, investigation of the importance of IFN β in the development of allergic sensitization is warranted by the findings here, and may ultimately lead to interventions to ameliorate the development of allergic airways disease.

METHODS

Animals

Specific pathogen-free 8-week-old female BALB/c mice were sourced commercially (Animal Resource Centre, Perth, WA, Australia), and bred under clean conditions at the Telethon Kids Institute Bio Resources Centre. Experiments were approved by the Telethon Kids Institutional Animal Ethics Committee.

Type I IFN and OVA administration

Mice were lightly anesthetized with isoflurane and administered vehicle only, 50 μ g OVA (Grade V chicken egg; Sigma-Aldrich, Sydney, NSW, Australia), 1000 IU IFN α 3 (Millipore, Temecula, CA, USA) and/or 1000 IU IFN β (Millipore, Temecula, CA, USA) intranasally in 50 μ L LPS free saline, as indicated.

OVA sensitization and OVA-IgE detection

OVA-sensitization of mice was performed by intraperitoneal injection, mice received 20 μ g OVA in 200 μ L 2.25 mg aluminum hydroxide (OVA-Alum; Serra, Germany). Serum OVA-IgE was detected using an Anti-Ovalbumin IgE (mouse ELISA kit (Cayman Chemical Company, Michigan, USA). Alternatively OVA-IgE antibody titers were determined using Passive Cutaneous Anaphylaxis. In brief, serum was serially 1 in 2 diluted in PBS and inoculated intradermally into the shaved backs of Wistar rats; 24 h later rats were challenged intravenously (i.v.) with 5 mg mL⁻¹ OVA in 1% Evans Blue (in PBS). Thirty minutes later, PCA titer was taken as the reciprocal of the highest dilution.

Bronchoalveolar lung lavage and sera collection

Twenty-four hours after the final intranasal OVA BALF was collected by flushing 1 mL of PBS into the lung. Cells were cytocentrifuged onto glass slides and stained with DIFF-Quik Stain Set 64851 (Lab Aids, Narrabeen, NSW, Australia). Sera were collected 24 h after the final aerosol by cardiac heart puncture, and stored at -20°C.

Trachea and ADLN cell preparations

Mouse tracheal and lymph node preparations have been described in detail previously.¹⁹ In brief, trachea or ADLN (upper

paratracheal and parathymic; five mice/group) were subjected to type IV collagenase digestion (1.5 mg mL⁻¹; Worthington Biochemical, Lakewood, NJ) with type I DNase (0.1 mg mL⁻¹; Sigma Aldrich) to prepare single-cell suspensions. All digestions and washes were performed in glucose sodium potassium buffer (GKN; 11 mmol L⁻¹ D-glucose, 5.5 mmol L⁻¹ KCl, 137 mmol L⁻¹ NaCl, 25 mmol L⁻¹ Na₂HPO₄, 5.5 mmol L⁻¹ NaH₂PO₄·2H₂O) as previously described.³⁷

FACS analysis and antibodies

Single-cell suspensions were FcR blocked routinely (2.4G2; BD Biosciences) prior to the addition of phenotyping antibodies. Airway and draining lymph node DC populations were identified using combinations of fluorochrome labeled mAbs, BD Pharmingen, to mouse I-A/I-E (2G9); CD11c (N418), CD11b (M1/70) and CD8a (53–6.7). All labeling was performed in GKN buffer containing 0.2% BSA for 30 min on ice. T-cell populations were identified in tracheal and ADLN digests using combinations of fluorochrome-labeled monoclonal antibodies specific for murine CD3, CD4, CD62L, CD44; CD3, CD4, CD25; or CD3, CD4, CD185, CD154 (BD Biosciences). A FOXP3 intracellular staining kit (eBiosciences, San Diego, CA) was used to determine intracellular Ki67 and FOXP3 staining. B cells were identified in ADLN using CD4 and CD8a negative gate, and B220 and IgE antibody staining as indicated. All Abs were used as direct conjugates to FITC, Phycoerythrin (PE), PE-Cy7, allophycocyanin, APC-Cy7, or biotin and streptavidin conjugated PE-Cy5 (BD Biosciences, San Jose, CA), as required. Appropriately matched IgG isotype controls (BD Pharmingen, Western Australia) and cytometer compensation settings adjusted using single-stained controls were used for each experiment. Samples were collected using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar Inc, Ashland, OR, USA).

Antigen uptake *in vivo*

Mice were lightly anesthetized with gaseous isoflurane and received 50 μ g OVA-conjugated Alexa Fluor 647 (OVA-A647; Molecular Probes, Eugene, Oregon) in 50 μ L endotoxin-free saline delivered intranasally. Mice were sacrificed at indicated time points after fluorescent OVA administration, and trachea and ADLN tissue were harvested.

Cytokine analysis

BALF was concentrated 15x with Nanosep centrifugal devices (3K, gray; PAL, Life Sciences) according to the manufacturer's instructions. Cytokine analysis was performed using the Bioplex Pro mouse Cytokine, 23-Plex Assay (Bio-Rad, NSW, Australia) according to manufacturer's protocol. In brief, premixed standards were reconstituted to 50 ng mL⁻¹ and serially diluted for an 8-point standard curve. Premixed beads (50 mL) were washed twice in a 96-well plate and 50 mL standard or sample added to each well. The plate was incubated at RT for 30 min with shaking. After washing,

beads were suspended in 125 μ L of Bio-Plex assay buffer and read on a Bioplex System (Bio-Rad, NSW, Australia).

Statistical analysis

Significant difference between treatment groups was determined by one-way ANOVA with Bonferroni's correction for multiple-testing. Significant difference between time points within groups was determined by an unpaired, two-tailed *t*-test. Statistical analysis was performed using Prism 6.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

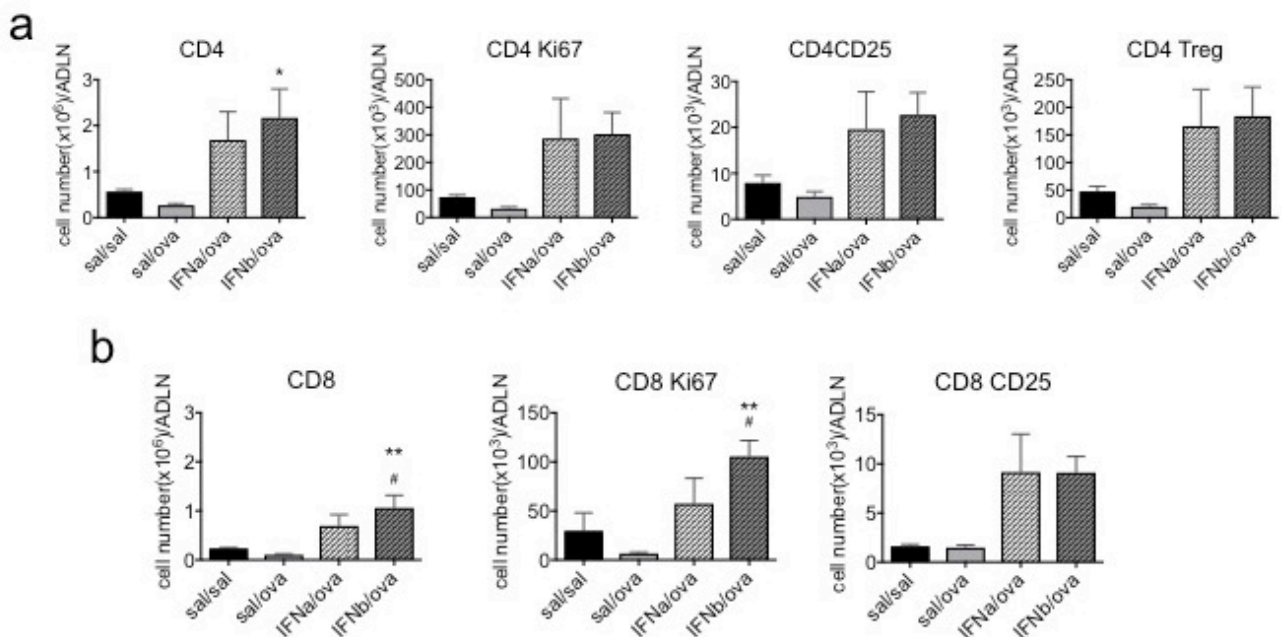
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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.



Supplementary Figure 1. Airways IFN β and allergen challenge enhances T cell responses in the ADLN. Mice received 1000IU IFN α , 1000IU IFN β , or saline intranasally at day 0, 3 and 9, with intranasal saline or OVA (50 μ g) on day 1, 4 and 10. ADLN were collected 24 h after the final OVA administration and digested to single cell suspension, and stained for flow cytometry. T cells were gated on CD4 or CD8 with subsequent gating on Ki67, CD25 $^+$ and FOXP3 $^+$. **(a)** CD4, CD4 Ki67 $^+$, CD4 CD25 $^+$ and CD4 Treg cell number. **(b)** CD8, and CD8 Ki67 $^+$ and CD8 CD25 $^+$ cell number. (n=5 mice/group; one-way ANOVA, *p<0.05. **p<0.005, compared to OVA pre-treatment, OVA challenge; #p<0.05. ##p<0.005, compared to saline pre-treatment, OVA-challenge; +p<0.05, ++p<0.005 compared to IFN α /OVA pretreatment, OVA challenge.)