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IL-28A (IFN-λ2) modulates lung DC function to promote Th1 immune skewing and suppress allergic airway disease

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IL-28 (IFN-λ) cytokines exhibit potent antiviral and antitumor function but their full spectrum of activities remains largely unknown. Recently, IL-28 cytokine family members were found to be profoundly down-regulated in allergic asthma. We now reveal a novel role of IL-28 cytokines in inducing type 1 immunity and protection from allergic airway disease. Treatment of wild-type mice with recombinant or adenovirally expressed IL-28A ameliorated allergic airway disease, suppressed Th2 and Th17 responses and induced IFN-γ. Moreover, abrogation of endogenous IL-28 cytokine function in IL-28Rα$^{-/-}$ mice exacerbated allergic airway inflammation by augmenting Th2 and Th17 responses, and IgE levels. Central to IL-28A immunoregulatory activity was its capacity to modulate lung CD11c^{+} dendritic cell (DC) function to down-regulate OX40L, up-regulate IL-12p70 and promote Th1 differentiation. Consistently, IL-28A-mediated protection was absent in IFN-γ$^{-/-}$ mice or after IL-12 neutralization and could be adoptively transferred by IL-28A-treated CD11c^{+} cells. These data demonstrate a critical role of IL-28 cytokines in controlling T cell responses *in vivo* through the modulation of lung CD11c^{+} DC function in experimental allergic asthma.

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INTRODUCTION

Allergic asthma is due to the failure of the immune system to develop tolerance to otherwise innocuous environmental aeroallergens (Holgate & Polosa, 2008). As a result, inappropriate Th2-mediated immune responses are induced that orchestrate the asthmatic response by promoting the production of IgE, the infiltration of lymphocytes and eosinophils, and the induction of airway inflammation and hyper-responsiveness (Umetsu & DeKruyff, 2006). Aeroallergen-directed Th17 responses can further contribute to this process by triggering the infiltration of neutrophils and the hyper-secretion of mucus (Holgate & Polosa, 2008). To prevent this from happening, the host employs a multitude of often complementary regulatory mechanisms that include the generation of Th1-specific immune responses that cross-regulate Th2 responses to allergen and/or the induction of regulatory T cell responses. The rate-limiting step in the generation of Th2-mediated allergic responses in the lung, and the induction of counter-

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regulatory Th1 or Treg responses, is the presentation of allergen by local DCs (van Rijt et al, 2005). This is influenced by the lung microenvironment and the presence of allergic or infectious insults that trigger the expression of key inflammatory cytokines such as thymic stromal lymphopoietin (TSLP) and IL-12 that modulate DC function and promote Th2 or Th1 cell differentiation, respectively. Still, the majority of factors responsible for controlling DC-mediated T helper cell polarization, especially in the context of allergic disease, remain incompletely understood.

Recently, a new family of cytokines known as interleukins-28/29, λ-interferons or type III interferons (IFN) was discovered. IL-28/29 cytokines represent an interesting evolutionary link between type I IFNs and the IL-10 family (Kotenko et al, 2003; Sheppard et al, 2003; Siren et al, 2005). They consist of three members in humans, denoted IL-28A (IFN-λ2), IL-28B (IFN-λ3), and IL-29 (IFN-λ1), and two members in mice (IL-28A and IL-28B) (Lasfar et al, 2006), all of which signal through a heterodimeric receptor consisting of IL-28Rα (IFN-λR1 or CRF2-12) responsible for ligand specificity, and IL-10Rβ (IL-10R2 or CRF2-4) shared with all other IL-10 family members. Although IL-10Rβ is ubiquitously expressed, IL-28Rα expression is restricted to cells of epithelial derivation including hepatocytes and myeloid lineage cells such as dendritic cells (DCs) and macrophages (Doyle et al, 2006; Mennechet & Uze, 2006).

IL-28 cytokine family members are produced by various cells including antigen presenting cells upon viral infection or Toll like receptor ligation (Doyle et al, 2006; Lauterbach et al, 2010; Siebler et al, 2007; Siren et al, 2005). It is now well established that IL-28 cytokines exhibit potent antiviral and anti-cancer activity. They inhibit viral replication (Robek et al, 2005), they up-regulate cytotoxic responses to virally infected cells (Ank et al, 2008) and they reduce tumour growth and metastases in mice (Numasaki et al, 2007). More recently, additional activities of IL-28 cytokine family members in the immune system have been suggested. For instance, IL-29-treated DCs induce proliferation of FoxP3-expressing regulatory T cells *in vitro* (Mennechet & Uze, 2006), whereas addition of IL-29 to human PBMC or T cell cultures *in vitro* inhibits the production of IL-5 and IL-13 (Dai et al, 2009; Jordan et al, 2007; Srinivas et al, 2008). Moreover, transgenic expression of IL-28A *in vivo* promotes the induction of Th1 over Th2 responses and the severity of ConA-induced liver injury (Siebler et al, 2007).

In humans, a strong link between low expression of IL-28 cytokines and severity of allergic asthma and allergic asthma exacerbations has been described. Asthmatic patients exhibit deficient induction of IL-28 and IL-29 in response to rhinovirus infection, and deficient IL-28/29 expression correlates with the severity of rhinovirus-induced asthma exacerbations and virus load in experimentally infected human volunteers (Contoli et al, 2006). Moreover, in the absence of detectable viral infection asthmatic patients with active disease still exhibit an inverse correlation between IL-28 and IL-29 mRNA levels and severity of the allergic response in the airways (Bullens et al, 2008). This raises the possibility that in addition to their role in antiviral immunity IL-28 cytokines may also modulate adaptive immune responses that underlie the pathogenesis of asthma. Here, we demonstrate that IL-28A promotes Th1 cell differentiation *in vivo* and suppresses Th2-mediated responses in the airways, and we identify IL-28 cytokines as new immunotherapeutic agents for the treatment of allergic airway disease.

RESULTS

Overexpression of IL-28A in the lung inhibits Th2 and Th17 responses and suppresses allergic airway disease

To explore the role of IL-28 cytokines in allergic airway disease, we used an established mouse model of allergen sensitization and challenge (Fig 1A). We first investigated the expression of IL-28A and IL-28B (97% identical in amino acid sequence) in the bronchoalveolar lavage fluid (BALF) during the development of allergic airway inflammation and found that, similarly to human patients, IL-28 cytokines were expressed at only low levels (Fig 1B). To up-regulate IL-28 levels and examine whether this has functional consequences in disease development, we constructed a recombinant replication-deficient adenovirus expressing IL-28A under control of the CMV promoter (AdIL-28) and applied it to the lung. This resulted in high levels of IL-28A production in the BALF of treated mice (Fig 1B), which profoundly impacted on allergic airway disease. We found that AdIL-28 but not Ad0 or vehicle control treatments significantly reduced eosinophil, neutrophil and lymphocyte cell numbers in the BALF (Fig 1C–D) and leukocyte infiltration in the perivascular and peribronchial areas of the lung (Fig 1E). This was accompanied by significantly decreased goblet cell metaplasia and hyper-secretion of airway epithelial mucus in the lung of AdIL-28-treated mice compared to controls (Fig 1F). Notably, effector Th2 and Th17 responses to OVA were also suppressed as demonstrated by the inhibition of IL-5, IL-10, IL-13 and IL-17 in the lung-draining mediastinal lymph nodes (MLNs), whereas the Th1 cytokine IFN-γ was up-regulated (Fig 1G). In contrast, IL-4 was not detectable in this assay (data not shown). Finally, protection from airway hyper-responsiveness (AHR) measured as metacholine-induced increases in total lung resistance was observed in mice treated with AdIL-28, whereas mock adenovirus (Ad0) or vehicle administration (PBS) had no effect (Fig 1H). Collectively, these findings demonstrate that high IL-28A levels in the lung effectively suppress the development of Th2 and Th17 cell mediated inflammatory responses and Th2-mediated allergic airway disease.

Intranasal administration of recombinant IL-28A effectively treats allergic airway disease in mice

Subsequently, we examined whether recombinant IL-28A (IL-28A) administered intranasally during OVA challenge (Fig 2A) would also be capable of suppressing Th2-mediated allergic airway disease and thus whether it could be therapeutically useful. Indeed, we found that IL-28A treatment effectively suppressed the number of eosinophils and neutrophils in the BALF (Fig 2B) although it had no effect on the number of lymphocytes (Fig 2C). IL-28A treatment also inhibited the

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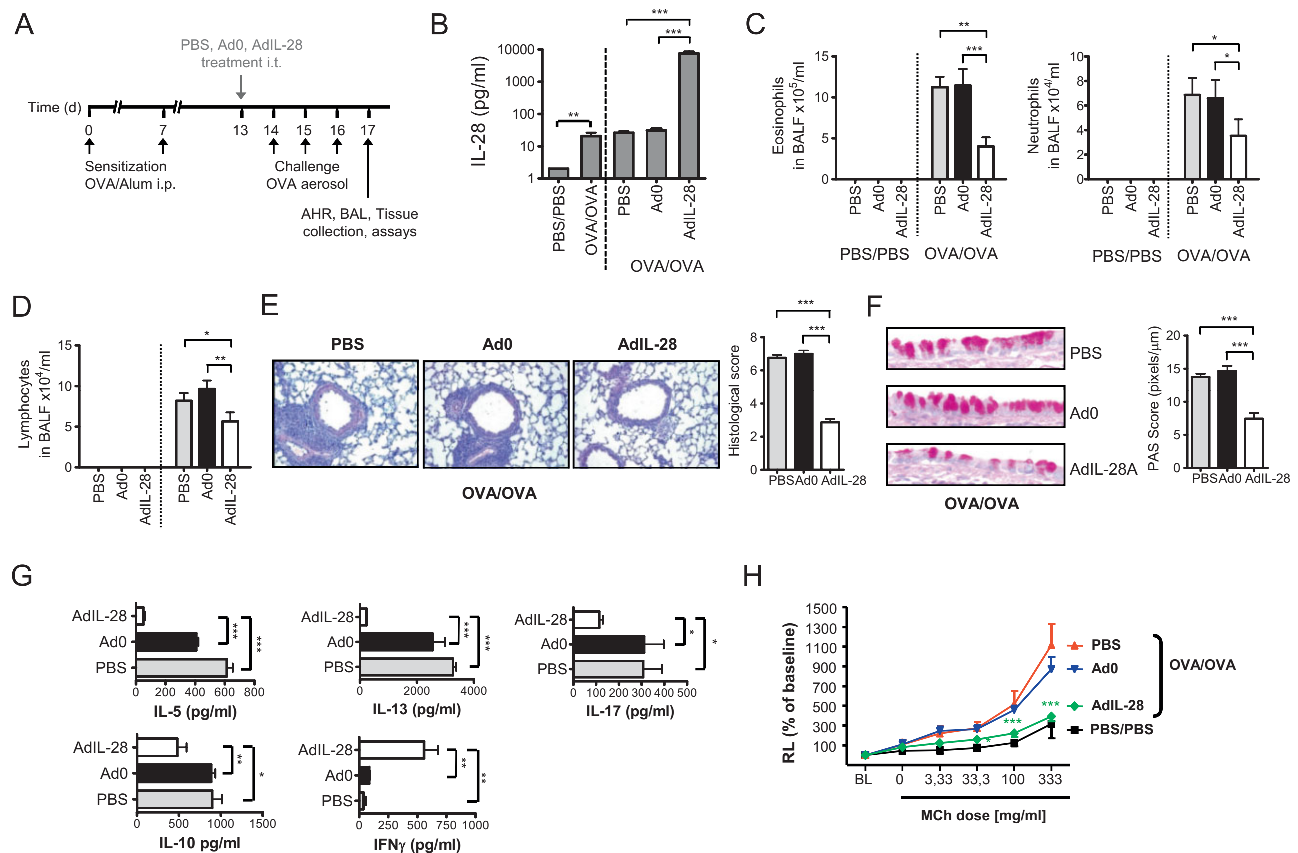

Figure 1. Adenoviral expression of IL-28A during allergen challenge suppresses the development of allergic airway disease.

A. Experimental protocol. C57BL/6 mice were subjected to vehicle (PBS), mock (Ad0) or IL-28A expressing adenovirus (AdIL-28) treatment in the lung and then challenged with aerosolized OVA (OVA/OVA) or PBS (PBS/PBS).

B. Total IL-28 levels in the BALF of lungs from PBS or OVA-sensitized and challenged mice in the presence of AdIL-28, Ad0 or PBS treatment. For the analysis of IL-28 levels, BALF was concentrated 10X as detailed in Materials and Methods section. Results are expressed as mean values ± SEM of 4–8 mice per group from two independent experiments.

C. BALF differential counts for eosinophils and neutrophils expressed as mean ± SEM of 10–14 mice per group from three independent experiments are shown.

D. BALF differential counts for lymphocytes expressed as mean ± SEM of 10–14 mice per group from three independent experiments are shown.

E. Histological assessment of lung inflammation in AdIL-28-treated mice. Hematoxylin and eosin stained lung sections and histological scoring expressed as mean values ± SEM from 10 mice per group are shown.

F. Histological assessment of mucus secretion in AdIL-28-treated mice. Periodic acid Schiff (PAS)-stained sections and morphometric analysis expressed as mean values ± SEM from 10 mice per group are shown.

G. Effector T cell responses in MLNs of OVA sensitized and challenged mice. Cytokine levels in supernatants of OVA-stimulated MLN cultures expressed as mean values ± SEM of five mice per group are shown. Data are representative of two independent experiments.

H. AHR measured as metacholine-induced increases in total lung resistance (RL) in mechanically ventilated mice. Data are expressed as mean values of percentage increase from baseline of the total RL ± SEM of eight mice per group from two independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

infiltration of leukocytes in the lung (Fig 2D) and led to the reduction of goblet cell metaplasia (Fig 2E). Notably, IL-28A-mediated suppression of disease severity was accompanied by a significant inhibition of IL-5, IL-13 and IL-17 in lung-draining MLNs and a marked up-regulation of IFN-γ (Fig 2F). Similar inhibition of Th2 and Th17 responses was also observed in purified lung $CD4^{+}$ T cells from IL-28A treated mice (Supporting Information Fig S1A–B). Finally, IL-28A treatment ameliorated lung function by reducing AHR in response to increasing doses of MCh (Fig 2G). These data support a potent therapeutic effect of recombinant IL-28A treatment in allergic airway disease.

IL-28Rα$^{-/-}$ mice exhibit augmented Th2 and Th17 responses and exacerbated allergic airway inflammation

To explore the role of endogenous IL-28 cytokine production in allergic airway disease, we took advantage of mice in which the *IL-28RA* gene encoding the alpha chain of the IL-28 receptor complex has been inactivated by homologous recombination (Ank et al, 2008). Using the OVA model of allergic airway inflammation (Fig 1A), we found that OVA-sensitized and -challenged IL-28Rα$^{-/-}$ mice exhibited a significant increase in eosinophilic cell infiltration in the BALF as compared to wild-type controls (Fig 3A, left panel). Although eosinophils constituted the main infiltrating cell population in the lung,

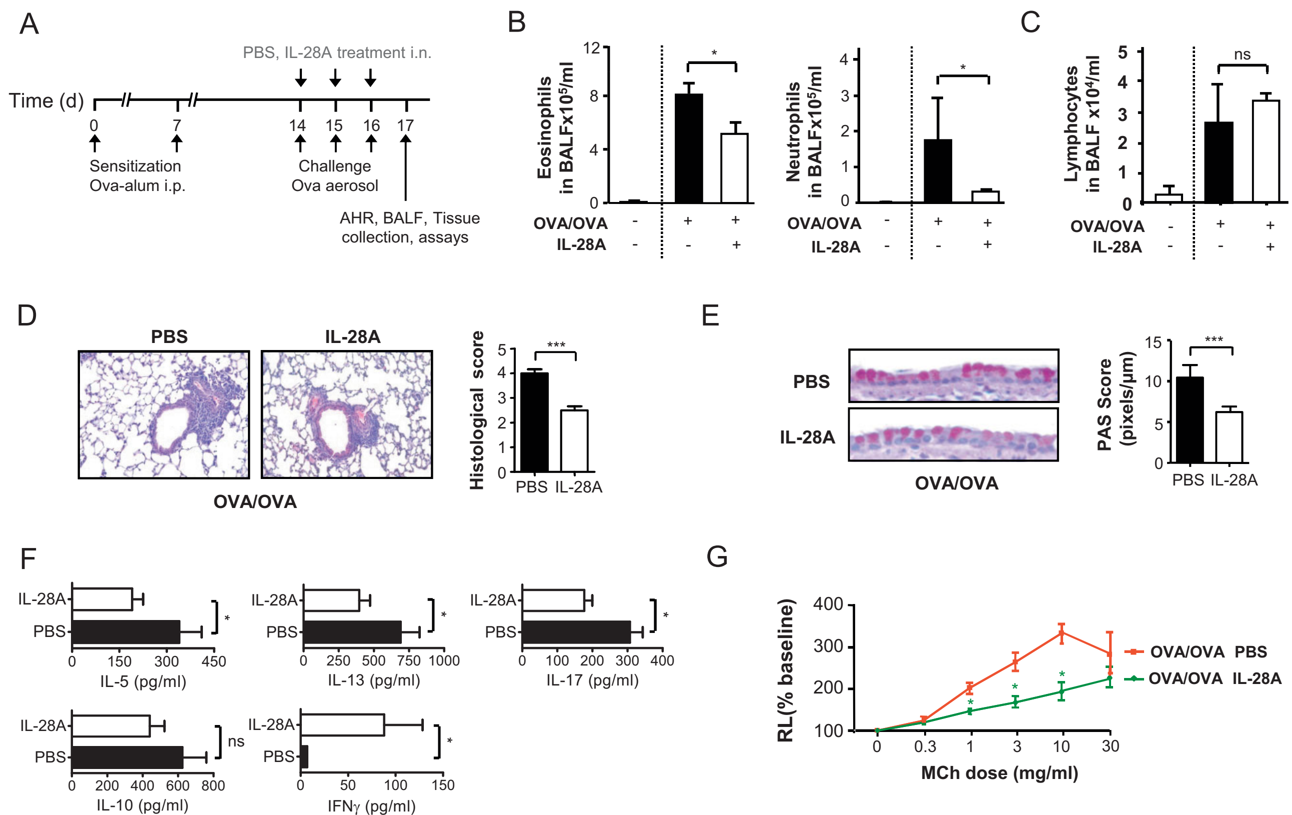

Figure 2. Treatment with recombinant IL-28A suppresses Th2/Th17 cytokine production and ameliorates allergic airway disease.

- **A.** Experimental protocol. BALB/c mice were challenged for three consecutive days with inhaled OVA in the absence (OVA/OVA + PBS) or presence of recombinant IL-28A (OVA/OVA + IL-28A). Control mice were challenged with PBS (PBS/PBS).
- **B.** BALF differential counts for eosinophils and neutrophils expressed as mean ± SEM of 3–4 mice per group. One representative of two independent experiments is shown.
- **C.** BALF differential counts for lymphocytes expressed as mean ± SEM of 3–4 mice per group. One representative of two independent experiments is shown.
- **D.** Histological assessment of lung inflammation in IL-28A-treated mice. Hematoxylin and eosin stained lung sections and histological scoring expressed as mean values ± SEM from six mice per group are shown.
- **E.** Histological assessment of mucus secretion in IL-28A-treated mice. PAS stained sections and morphometric analysis expressed as mean values ± SEM from six mice per group are shown.
- **F.** Effector T cell responses in MLNs of OVA sensitized and challenged mice. Cytokine levels are expressed as mean values ± SEM in supernatants of OVA-stimulated MLN cultures of 10–12 mice per group from two independent experiments.
- **G.** AHR measured as metacholine-induced increases in total lung resistance (RL) in anesthetized BALB/c mice. Data are expressed as mean values of percentage increase from baseline of the total RL ± SEM of five mice per group. One representative of three independent experiments is shown. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

an increase in neutrophils (Fig 3A, right panel) and a tendency for increased lymphocytes (Fig 3B) was also observed in IL-28R$\alpha^{-/-}$ mice. This was further accompanied by significantly enhanced inflammatory infiltrates in the lung (Fig 3C) and goblet cell metaplasia in the airways (Fig 3D) of IL-28R$\alpha^{-/-}$ mice. Notably, effector Th2 and Th17 cell responses against OVA were also increased in IL-28R$\alpha^{-/-}$ mice. MLN cells from IL-28R$\alpha^{-/-}$ mice exhibited significantly higher levels of IL-5, IL-13 and IL-17 than their wild-type counterparts whereas IFN-γ levels were very low, due to the strong Th2 skewing of this model, and not affected (Fig 3E). Similar observations were also made when CD4^{+} T cells from the lung of IL-28R$\alpha^{-/-}$ mice were analysed (Supporting Information Fig S2A–C). Although IL-4 was not detectable in MLN cultures, it was readily produced in CD4^{+} T cell cultures from the lung and significantly up-regulated in IL-28R$\alpha^{-/-}$ mice (Supporting Information Fig S2A). Finally, IL-28R$\alpha^{-/-}$ mice exhibited increased IgE levels in the serum compared to wild-type controls (Fig 3F).

In contrast, AHR of OVA-sensitized and challenged IL-28R$\alpha^{-/-}$ mice was only marginally affected at saline and low MCh doses but not thereafter (Fig 3G), suggesting the influence of additional non-inflammatory parameters to this response. Indeed, remodelling changes such as increased collagen deposition around the bronchi were also observed in IL-28R$\alpha^{-/-}$ mice (Supporting Information Fig S2D). Taken together, these data indicate that endogenously produced IL-

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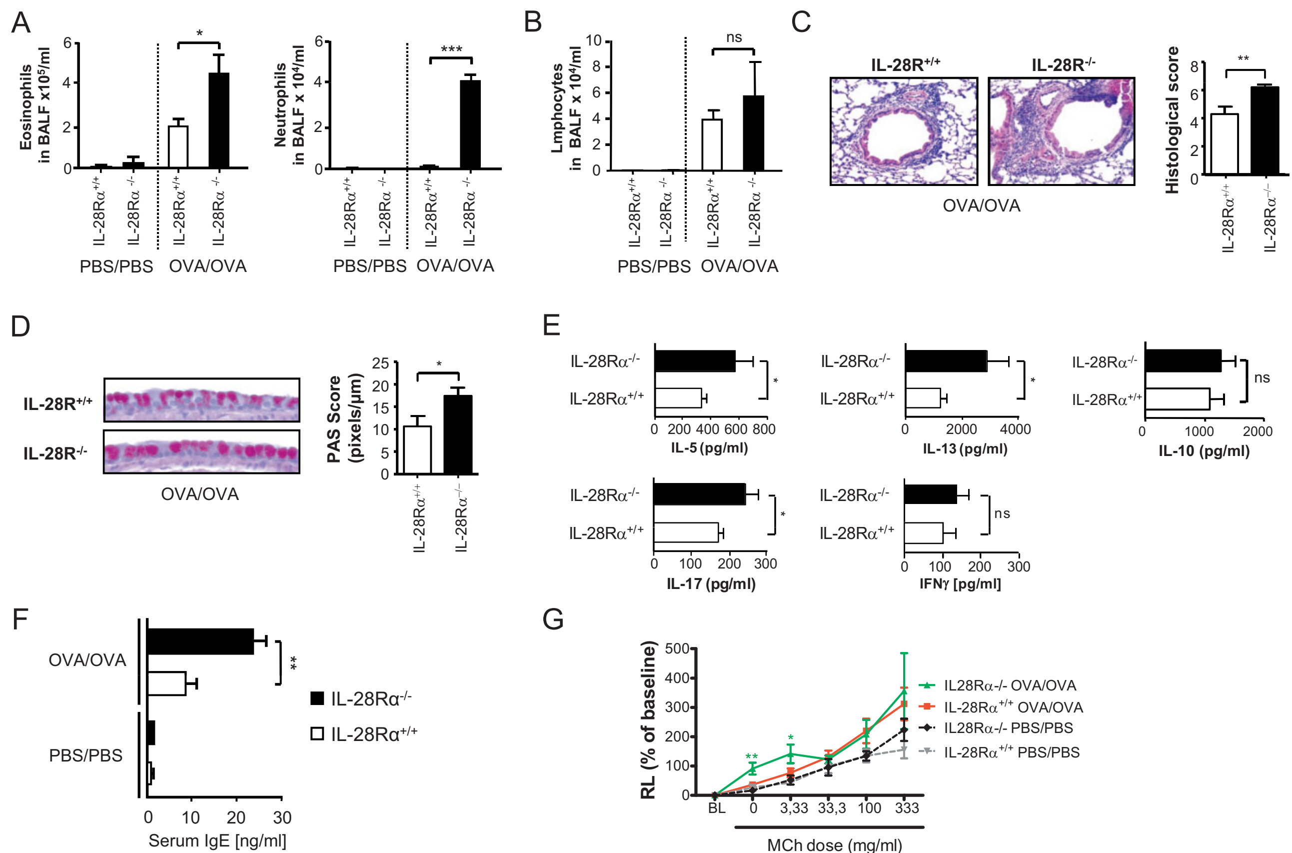

Figure 3. IL-28Rα-deficient mice develop increased Th2/Th17 responses and Th2/Th17-mediated allergic airway inflammation.

- **A.** BALF differential counts of eosinophils and neutrophils expressed as mean ± SEM of 3–4 mice per group. One representative from three independent experiments is shown.
- **B.** BALF differential counts of lymphocytes expressed as mean ± SEM of 3–4 mice per group. One representative from three independent experiments is shown.
- **C.** Histological assessment of lung inflammation in IL-28Rα$^{+/+}$ and IL-28Rα$^{-/-}$ mice. Hematoxylin and eosin stained lung sections and histological scoring expressed as mean values ± SEM from 5 to 7 mice per group are shown.
- **D.** Histological assessment of mucus secretion in IL-28Rα$^{+/+}$ and IL-28Rα$^{-/-}$ mice. PAS stained sections and morphometric analysis expressed as mean values ± SEM from 5 to 7 mice per group are shown.
- **E.** Effector T cell responses in MLNs of OVA sensitized and challenged mice. Cytokine levels in supernatants of OVA-stimulated MLN cultures expressed as mean values ± SEM of seven mice per group from two independent experiments are shown.
- **F.** Increased total IgE levels in the serum of IL-28Rα$^{-/-}$ mice as compared to IL-28Rα$^{+/+}$ littermates after OVA sensitization and challenge. Data represent mean values ± SEM.
- **G.** AHR measured as metacholine-induced increases of total lung resistance (RL) in mechanically ventilated IL-28Rα$^{+/+}$ and IL-28Rα$^{-/-}$ mice. Data are expressed as mean values of percentage increase from baseline of the total RL ± SEM from 10 mice per group pooled from two independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

28 cytokines are involved in damping down allergic airway inflammation and mucus hyper-secretion in mice.

IL-28 cytokines drive Th1 differentiation *in vivo*

Inhibition of effector Th2 responses and induction of IFN-γ seem to be central to IL-28A-mediated suppression of allergic airway disease. To get insight into this process, we examined the ability of IL-28 cytokines to modulate T helper cell differentiation during a primary immune response *in vivo*. We found that during primary immunization with OVA in alum, IL-28Rα$^{-/-}$ mice developed markedly enhanced Th2 and Th17 responses to OVA than their wild-type counterparts characterized by increased production of IL-4, IL-5, IL-13 and IL-17 (Fig 4A). At the same time, IL-28Rα$^{-/-}$ mice exhibited impaired IFN-γ production in response to OVA (Fig 4B) or the immunodominant CD4^{+} T cell specific OVA peptide OT-II (Fig 4C), suggesting that endogenous IL-28 cytokines (IL-28A and/or IL-28B) are critically involved in promoting CD4^{+} T cell differentiation to a Th1 phenotype. IL-28-induced Th1 cell differentiation appears to be a general aspect of the biology of IL-28 cytokines as mice immunized with OVA emulsified in Complete Freud's adjuvant (OVA/CFA), a potent Th1-inducing adjuvant, also exhibited reduced Th1 and enhanced Th2 responses to OVA (Supporting Information Fig S3A–B). On the contrary, IL-28A overexpression during primary immunization with OVA in alum exerted exactly the opposite effect by increasing IFN-γ and reducing IL-5, IL-13

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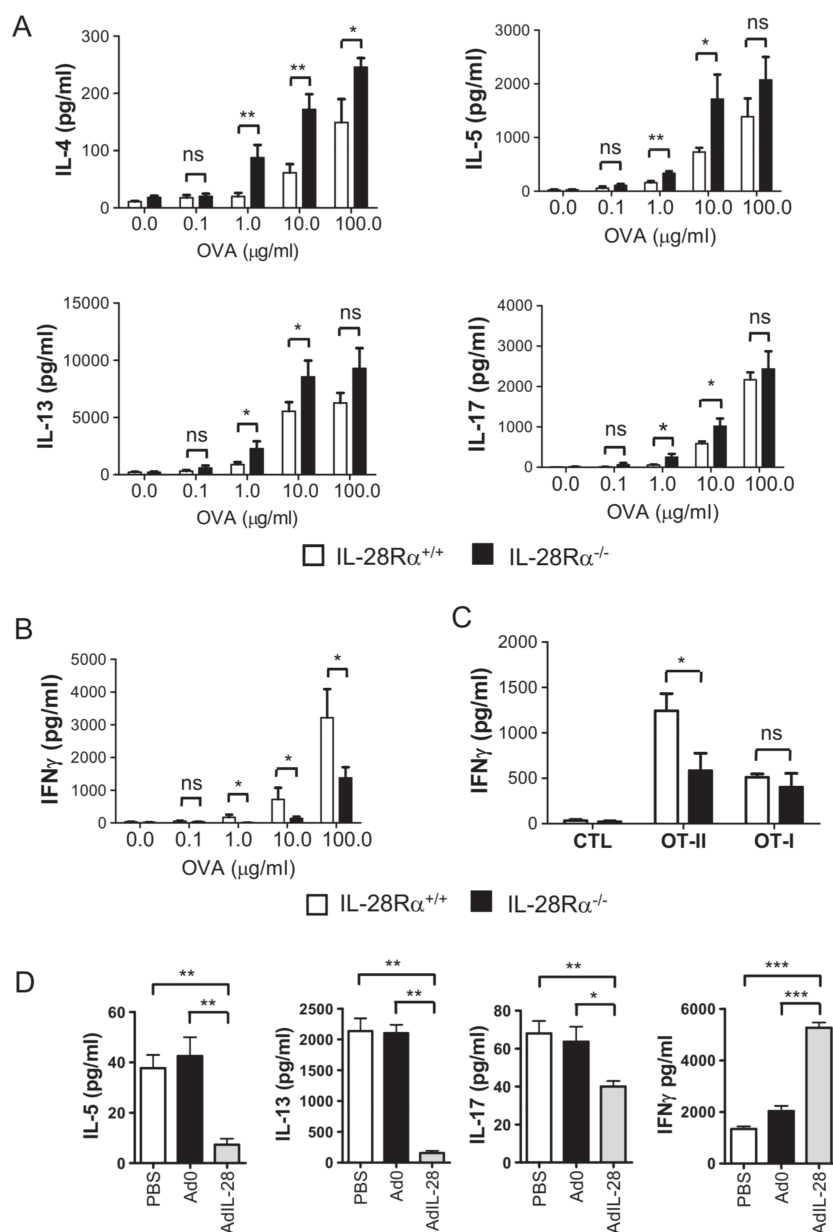

Figure 4. IL-28 signalling is required for skewing T helper cell differentiation to a Th1 cytokine profile.

- **A.** Primary Th2 cell responses in OVA/alum immunized IL-28Rα$^{+/+}$ and IL-28Rα$^{-/-}$ mice 6 days post-immunization. Cytokine levels in supernatants of OVA-stimulated splenocyte cultures expressed as mean values ± SEM of five mice per group are shown. One representative of two independent experiments is shown.
- **B.** Primary Th1 cell responses in OVA/alum immunized IL-28Rα$^{+/+}$ and IL-28Rα$^{-/-}$ mice 6 days post-immunization. Levels of IFN-γ in supernatants of OVA-stimulated splenocyte cultures expressed as mean values ± SEM of five mice per group are shown. One representative of two independent experiments is shown.
- **C.** IFN-γ production from CD4$^+$ and CD8$^+$ T cells of OVA/alum immunized IL-28Rα$^{+/+}$ and IL-28Rα$^{-/-}$ mice 6 days post-immunization. Levels of IFN-γ in supernatants of OT-II and OT-I-stimulated splenocyte cultures expressed as mean values ± SEM of five mice per group are shown. One representative of two independent experiments is shown.
- **D.** Effect of IL-28A expressing adenovirus (AdIL-28) treatment on primary Th2 cell responses of OVA/alum immunized C57BL/6 wild-type mice. AdIL-28, Ad0 or vehicle control (PBS) were administered to mice 1 day pre-immunization and T cell responses assessed 6 days post-immunization. Cytokine levels in supernatants of OVA-stimulated splenocyte cultures expressed as mean values ± SEM of five mice per group are shown. One representative of two independent experiments is shown. $^*p < 0.05$; $^{**}p < 0.01$; ns, non-significant.

and IL-17 production (Fig 4D). Taken together, these findings suggest that IL-28 cytokines act as inhibitors of Th2 and Th17 cell differentiation by favouring Th1 responses *in vivo*.

IL-28A reprograms lung DCs to promote type 1 responses and inhibit Th2 cell development *in vivo*

To understand how IL-28A controls T helper cell differentiation and Th2-mediated allergic airway disease, and explain the strong Th1-polarizing capacity of IL-28A *in vivo*, we assessed the expression of IL-28Rα on lung immune cells. Using cell sorting and quantitative PCR, we found that lung CD11c$^+$ DCs and cultured bone marrow derived DCs expressed high levels of IL-28Rα mRNA (Fig 5A). Consistently, alveolar macrophages and DC-like cells present in the lung parenchyma stained positive for IL-28Rα protein expression by immunohistochemistry (Fig 5B), while peribronchial inflammatory cell infiltrates were negative. In contrast, sorted CD4$^+$ T cells from the lung or the spleen, or *in vitro* differentiated T helper cells from the spleen, did not express significant levels of IL-28Rα mRNA (Fig 5A) and IL-28A treatment did not affect their activation or differentiation to T helper subsets (Supporting Information Fig S5A–D).

We then asked whether IL-28A treatment could affect lung CD11c$^+$ DC function. First, we found that IL-28A-treated lung CD11c$^+$ DCs cultured in the presence of LPS expressed significantly higher levels of T-bet, a transcription factor recently described to confer a Th1 polarizing phenotype to DCs (Lugo-Villarino et al, 2003; Wang et al, 2006), than control cells (Fig 5C and Supporting Information Fig S4B). This was disease-relevant as many allergens trigger TLR4 activation

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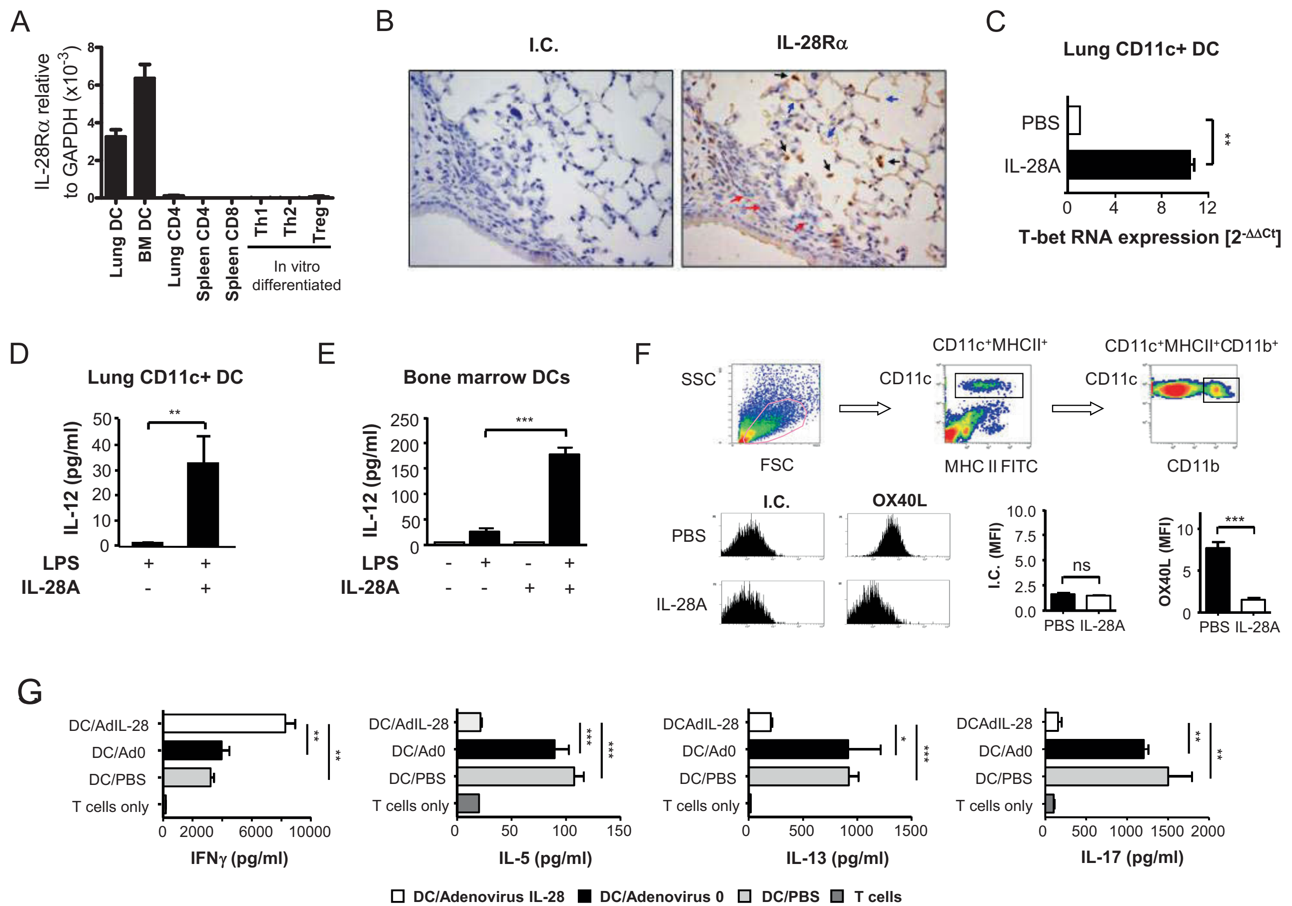

Figure 5. IL-28A modulates DC function to up-regulate IL-12 and promote Th1 over Th2/Th17 cell development.

A. Real-time PCR analysis of IL-28Rα mRNA levels of purified CD11c$^+$ DCs and CD4$^+$ T cells from the lung or spleen, or *in vitro* differentiated Th1, Th2 and Treg cells. Results are expressed as mean levels ± SEM of IL-28Rα expression relative to GAPDH.

B. Immunohistochemical analysis of IL-28Rα in lung sections from OVA sensitized and challenged mice. IL-28Rα was detected in alveolar macrophages and DC-like cells (black arrows) and alveolar epithelial cells (blue arrows) whereas inflammatory infiltrates were mostly negative (red arrows). Photomicrographs of lung sections stained with antibody control (I.C.) or IL-28Rα antibody are shown. Specific staining is depicted in brown; nuclei are stained blue with hematoxylin.

C. T-bet expression of lung CD11c$^+$ cells from naïve BALB/c mice. Purified lung CD11c$^+$ cells were cultured in the presence or absence of IL-28A for 24 h and T-bet mRNA levels assessed by real-time PCR. Results are expressed as mean fold induction ± SEM relative to untreated cells using the $2^{-\Delta\Delta Ct}$ method.

D. IL-12 induction from lung CD11c$^+$ cells after IL-28A treatment *in vivo*. Lung CD11c$^+$ cells isolated from PBS and IL-28A treated naïve C57BL/6 mice were cultured with LPS (1 µg/ml) for 24 h. IL-12p70 levels in cell supernatants are expressed as mean values ± SEM of four mice per group.

E. IL-12 induction from BM derived CD11c$^+$ cells after IL-28A treatment *in vitro*. BM DCs were cultured with LPS (1 µg/ml) in the presence or absence of IL-28A (100 ng/ml) for 24 h. IL-12p70 levels in cell supernatants are expressed as mean values ± SEM and are representative of three independent experiments.

F. FACS analysis of lung CD11c$^+$MHCII$^+$CD11b$^+$ DCs after IL-28A treatment of CD11c$^+$MHCII$^+$CD11b$^+$ DCs from whole lung digests of OVA sensitized mice were analysed for the expression of OX40L. Results are expressed as mean fluorescence intensity (MFI) ± SEM of five mice per group. Isotype control (I.C.) MFI levels are also shown.

G. Defective Th2-priming capacity of lung CD11c$^+$MHCII$^+$CD11b$^+$ DCs after AdIL-28 treatment. Lung CD11c$^+$MHCII$^+$CD11b$^+$ DCs sorted from AdIL-28 (DC/AdIL-28), Ad0 (DC/Ad0) or vehicle control treatment (DC/PBS) were cultured with purified CD4$^+$ T cells from OT-II transgenic mice for 7 days and supernatants assessed by ELISA. Cytokine levels expressed as mean ± SEM from four mice per group are shown. One representative of two independent experiments is shown. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, non-significant.

either because of structural mimicry with LPS or contamination by LPS itself (Hammad et al, 2009; Trompette et al, 2009). Second, IL-28A induced the production of significant levels of IL-12p70, a key cytokine mediating Th1 development, in both lung CD11c$^+$ DCs and bone marrow DCs cultured with LPS (Fig 5D–E). This is particularly important as lung CD11c$^+$ DCs are programmed to induce Th2 polarization and make little IL-12 even in response to LPS stimulation (Dodge et al, 2003). Finally, IL-28A down-regulated the expression of OX40 ligand (OX40L) on CD11c$^+$ DCs (Fig 5F and Supporting Information Fig S6A), a key costimulatory signal for optimal Th2 priming and memory induction *in vivo* (Jenkins et al, 2007).

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Functionally, IL-28A-mediated modulation of CD11c$^+$ DCs profoundly affected their ability to induce Th2 and Th17 cell responses *in vivo*. Sorted lung CD11c$^+$MHCII$^+$CD11b$^+$ DCs from AdIL-28-treated mice, a major and critical DC subset for the development of allergic responses in the lung (Julia et al, 2002; van Rijt et al, 2005), had an increased capacity to drive Th1 and a reduced capacity to drive Th2 or Th17 differentiation of OVA-specific naive CD4$^+$ T cells from OT-II transgenic mice when compared to lung CD11c$^+$MHCII$^+$CD11b$^+$ DCs from Ad0-treated or vehicle control mice (Fig 5G). Furthermore, immunization of naïve mice with IL-28A-treated BM DC pulsed with OVA profoundly inhibited the generation of Th2 and Th17 responses while Th1 polarization was enhanced (Supporting Information Fig S6B–C). Overall, these findings suggest that through the induction of IL-12, and possibly through the down-regulation of OX40L, IL-28A reprograms CD11c$^+$ DCs to promote type 1 responses and inhibit Th2 and Th17 cell development and associated pathology *in vivo*.

Adoptive transfer of IL-28A-treated CD11c$^+$ DCs promotes immune shifting to a Th1 cytokine profile and suppresses experimental allergic airway disease

To investigate further the possibility that CD11c$^+$ DCs are involved in the mode of action of IL-28A treatment in allergic airway disease, we performed adoptive transfer experiments of DCs from AdIL-28- or control-treated mice. Accordingly, we

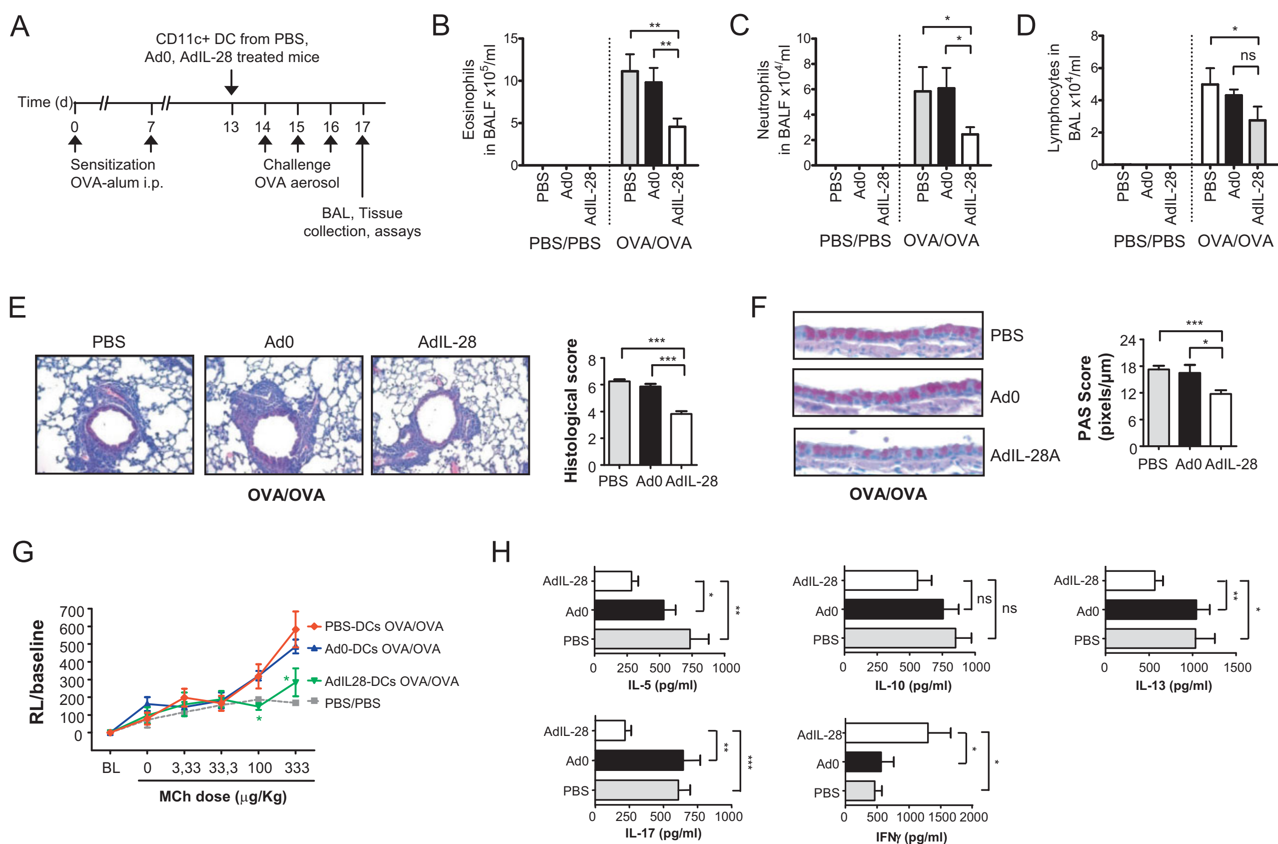

Figure 6. Adoptive transfer of CD11c$^+$ DCs from IL-28A-treated mice mediates Th1 immune skewing and suppresses allergic airway disease.

- **A.** Experimental protocol used for adoptive transfer of CD11c$^+$ DC. C57BL/6 mice received OVA-pulsed spleen CD11c$^+$ DCs from AdIL-28, Ad0 or vehicle control (PBS)-treated mice 1 day before OVA challenge.
- **B.** BALF differential counts for eosinophils expressed as mean ± SEM of 7–9 mice per group pooled from two independent experiments are shown.
- **C.** BALF differential counts for neutrophils expressed as mean ± SEM of 7–9 mice per group pooled from two independent experiments are shown.
- **D.** BALF differential counts for lymphocytes expressed as mean ± SEM of 7–9 mice per group pooled from two independent experiments are shown.
- **E.** Histological assessment of lung inflammation after adoptive transfer of CD11c$^+$ DC. Hematoxylin and eosin stained lung sections and histological scoring expressed as mean values ± SEM from 6 to 7 mice per group are shown.
- **F.** Histological assessment of mucus secretion after adoptive transfer of CD11c$^+$ DC. PAS stained sections and morphometric analysis expressed as mean values ± SEM from 6 to 7 mice per group are shown.
- **G.** AHR measured as metacholine-induced increases of total lung resistance (RL) in mechanically ventilated mice. Data are expressed as mean values of percentage increase from baseline of the total lung resistance ± SEM from six mice per group. One representative of two independent experiments is shown.
- **H.** T cell responses in MLNs of OVA sensitized and challenged mice after adoptive transfer of CD11c$^+$ DCs. Cytokine levels are expressed as mean values ± SEM in supernatants of OVA-stimulated MLN cultures of 7–9 mice per group from two independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, non-significant.

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purified CD11c$^+$ DCs from vehicle control (PBS/DC), Ad0 (Ad0/DC) or AdIL-28-treated mice (AdIL-28/DC), pulsed them with allergen and administered them to mice 1 day before OVA challenge (Fig 6A). Notably, we found that CD11c$^+$ DCs from AdIL-28-treated mice, but not Ad0- or PBS-treated mice, potently transferred the suppression mediated by IL-28A on Th2-mediated allergic airway disease. AdIL-28/DCs significantly reduced the severity of allergic airway inflammation (Fig 6B–E), decreased goblet cell metaplasia in lung tissue (Fig 6F) and prevented the development of AHR (Fig 6G) compared to Ad0- or PBS-treated mice. This was associated with up-regulation of IFN-γ and suppression of effector Th2 and Th17 responses in MLNs (Fig 6H). Similar results were also observed when CD11c$^+$ DCs from AdIL-28- but not Ad0- and PBS-treated mice were administered to mice 1 day before OVA sensitization (Supporting Information Fig S7), revealing an inhibitory effect of AdIL-28/DC in effective Th2 priming induced by OVA/alum immunization as well. Therefore, modulation of CD11c$^+$ DC function to inhibit Th2 and Th17 responses is a critical step involved in IL-28A-mediated suppression of allergic airway disease.

IL-28A-mediated suppression is critically dependent on IFN-γ and IL-12

As the above data suggested that IL-28A up-regulates type 1 cytokine responses in the lung through the modulation of DC function, we next examined whether IFN-γ is involved in the immunosuppressive mode of action of IL-28A in allergic airway disease. Using IFN-$\gamma^{-/-}$ mice, we found that the therapeutic benefit of recombinant IL-28A administration in the effector phase of allergic airway disease was completely reversed in the absence of IFN-γ. There was no significant difference in eosinophil, neutrophil or lymphocyte numbers in the BALF (Fig 7A–B) and no difference in leukocytic cell infiltration (Fig 7C) and goblet cell metaplasia (Fig 7D) in the lung of vehicle-treated and IL-28A-treated knockout mice. Moreover, AHR induced in response to metacholine challenge was comparable between IL-28A and vehicle-treated mice (Fig 7E). In MLN cultures of IFN-$\gamma^{-/-}$ mice, IL-28A treatment had no effect in IL-13 or IL-17 production although it slightly but significantly reduced IL-10 production (Fig 7F). There was also a tendency for reduced IL-5 production (Fig 7F), although this was not significant ($p = 0.0510$).

We further investigated whether IL-12, up-regulated in response to IL-28A in DCs, could be involved in driving IFN-γ production and associated disease suppression. We found that administration of a neutralizing antibody directed against the p40 subunit of IL-12, but not its isotype control, completely reversed IL-28A-mediated suppression of allergic airway disease. Anti-IL-12p40 treatment restored the numbers of eosinophils and neutrophils in the BALF (Fig 7G–H), the extent of leukocytic cell infiltration in the lung (Fig 7I) and the metaplasia of bronchial epithelial cells to goblet cells in the airways (Fig 7J). Anti-IL-12p40 treatment also abrogated the induction of IFN-γ after IL-28A treatment and restored the magnitude of OVA-specific Th2 and Th17 responses in MLNs (Fig 7K). These findings identify IFN-γ and IL-12 as critical mediators of IL-28A immunosuppressive function in allergic airway disease.

DISCUSSION

IL-28 family cytokines or λ-interferons are the latest addition to the class II cytokine family (Kotenko et al, 2003; Sheppard et al, 2003). They exhibit potent antiviral and anti-tumour function but their full spectrum of activities remains poorly characterized. In the present study, we have uncovered a new role of IL-28 cytokines as key regulators of adaptive immunity and allergic airway disease. By taking advantage of genetically engineered mice deficient in IL-28R signalling, IL-28A gene transfer and recombinant IL-28A administration, we show that IL-28 cytokines are critical for driving Th1 differentiation *in vivo* while limiting Th2 and Th17 responses in the airways. Consistently, IL-28R$\alpha^{-/-}$ mice develop exacerbated allergic airway inflammation associated with augmented eosinophilic and neutrophilic cell infiltration in the lung whereas wild-type mice treated with IL-28A exhibit markedly reduced Th2 and Th17 responses and Th2/Th17-mediated allergic airway disease. Although our findings are based on IL-28R$\alpha^{-/-}$ mice and IL-28A treatment, they are likely to pertain to all IL-28 cytokines as IL-28A shares 96–97% identity in amino acid sequence with IL-28B and 81% identity with IL-29, and as all cytokines signal through a common receptor complex (Kotenko et al, 2003; Sheppard et al, 2003).

Key to the suppressive action of IL-28A was immune shifting from a Th2/Th17 to a Th1 cytokine profile and induction of IFN-γ. Accordingly, T cells from wild-type mice treated with recombinant IL-28A exhibited significantly reduced IL-4, IL-5, IL-13 and IL-17 production and markedly up-regulated IFN-γ, whereas T cells from IL-28R$\alpha^{-/-}$ mice exhibited significantly increased levels of IL-4, IL-5, IL-13 and IL-17, and augmented IgE production. Moreover, T cell-derived IFN-γ was essential for IL-28A-mediated inhibition of Th2 and Th17 responses and allergic airway disease as demonstrated by the lack of protection in IFN-$\gamma^{-/-}$ mice. This is in agreement with the potent inhibitory activity of IFN-γ in several aspects of the allergic response in the lung including Th2 and Th17 cytokine production, eosinophilia, neutrophilia, goblet cell metaplasia and AHR (Cohn et al, 1999; Durrant et al, 2009). Notably, IL-28A-mediated Th1 skewing and IL-28A-mediated suppression of allergic airway disease were both dependent on IL-12 as neutralization of this cytokine abrogated the induction of IFN-γ and reversed the protective action of IL-28A in the development of allergic airway inflammation. These findings demonstrate that the activation of the IL-12-IFN-γ axis is a rate-limiting step involved in the suppressive function of IL-28A.

IL-28A-induced immune shifting to a Th1 cytokine profile was mediated through the modulation of conventional DC function rather than direct effects on T cells. In agreement with previous reports (Ank et al, 2008; Doyle et al, 2006; Witte et al, 2009), but in disagreement with some others (Dai et al, 2009), we found that neither highly purified CD4$^+$ or CD8$^+$ T cells nor differentiated Th1 or Th2 cells express significant levels of IL-28Rα mRNA, and neither of them responds to IL-28 treatment to up-regulate known IL-28R target genes (data not shown). Moreover, neither T cell differentiation induced *in vitro* in the absence of DCs nor cytokine production of already differentiated Th0, Th1 or Th2 cells is affected by IL-28A treatment. In contrast, both CD11c$^+$ lung and bone marrow derived DCs, as

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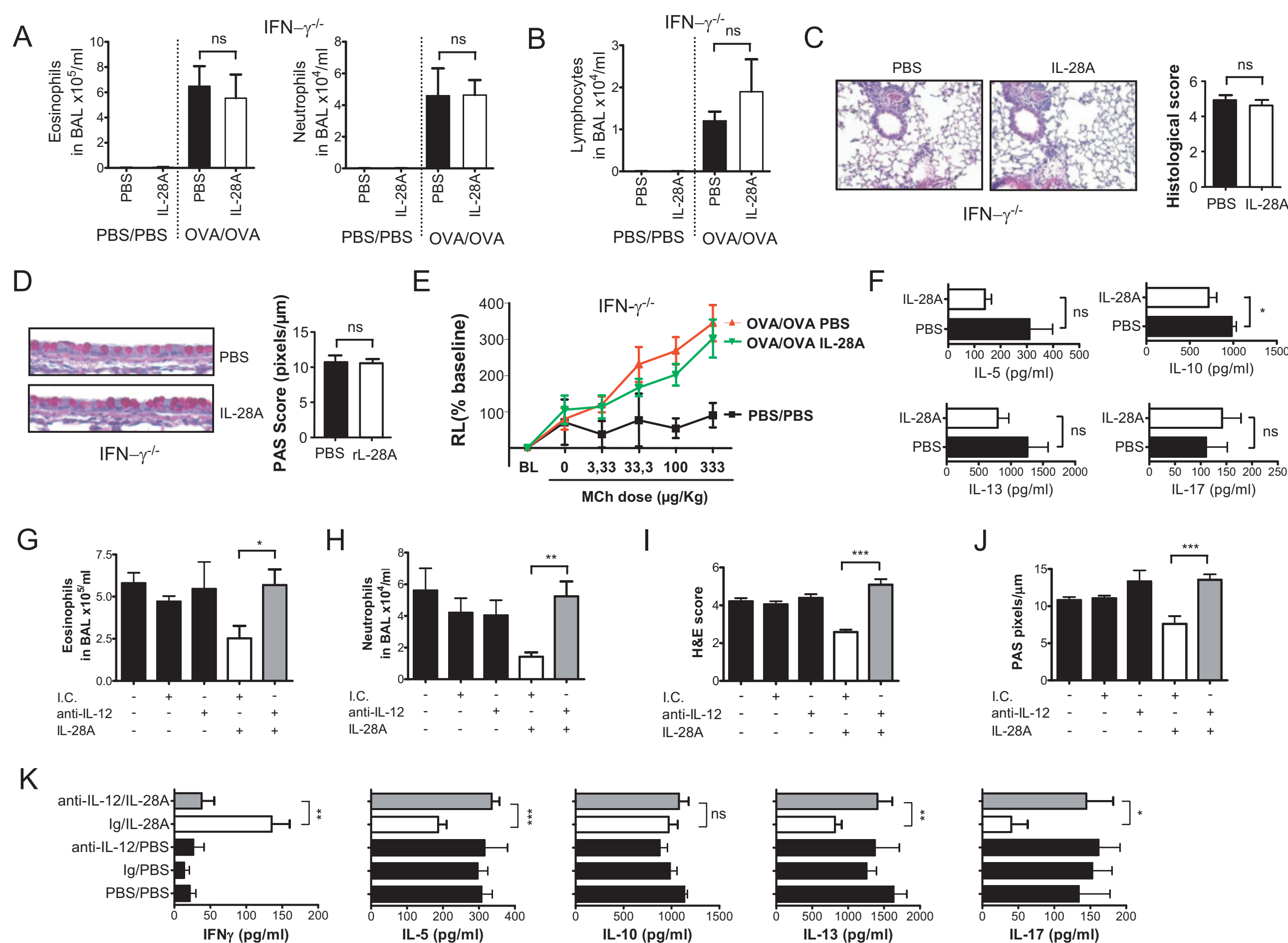

Figure 7. IFN-γ and IL-12 are required for IL-28A-mediated suppression of allergic airway disease. IFN-$\gamma^{-/-}$ and wild-type C57BL/6 mice were subjected to PBS or OVA sensitization and challenge in the presence of IL-28A (IL-28A) or vehicle control (PBS) treatment as in Fig 2A.

- **A.** BALF differential counts of IFN-$\gamma^{-/-}$ mice for eosinophils and neutrophils expressed as mean ± SEM of six mice per group from two independent experiments are shown.
- **B.** BALF differential counts of IFN-$\gamma^{-/-}$ mice for lymphocytes expressed as mean ± SEM of six mice per group from two independent experiments are shown.
- **C.** Histological assessment of lung inflammation in OVA sensitized and challenged IFN-$\gamma^{-/-}$ mice. Hematoxylin and eosin stained lung sections and histological scoring expressed as mean values ± SEM from five mice per group are shown.
- **D.** Histological assessment of mucus secretion in OVA sensitized and challenged IFN-$\gamma^{-/-}$ mice. PAS stained sections and morphometric analysis expressed as mean values ± SEM from five mice per group are shown.
- **E.** AHR measured as metacholine-induced increases in total lung resistance (RL) in mechanically ventilated IFN-$\gamma^{-/-}$ mice. Data are expressed as mean values of percentage increase from baseline of the total lung resistance ± SEM of five mice per group. One representative of two independent experiments is shown.
- **F.** T cell responses in MLNs of OVA sensitized and challenged IFN-$\gamma^{-/-}$ mice. Cytokine levels in supernatants of OVA-stimulated MLN cultures expressed as mean values ± SEM of five mice per group are shown. Data are representative of two independent experiments.
- **G.** BALF differential counts of eosinophils from IL-28A (IL-28A) or vehicle control (PBS) treated OVA challenged wild-type mice in the presence of anti-IL-12p40 (anti-IL-12) or isotype control (Ig) antibody. Results are expressed as mean ± SEM of six mice per group.
- **H.** BALF differential counts of neutrophils from IL-28A (IL-28A) or vehicle control (PBS) treated OVA challenged wild-type mice in the presence of anti-IL-12p40 (anti-IL-12) or isotype control (Ig) antibody. Results are expressed as mean ± SEM of six mice per group.
- **I.** Histological assessment of lung inflammation in IL-28A (IL-28A) or vehicle control (PBS) treated OVA challenged wild-type mice in the presence of anti-IL-12p40 (anti-IL-12) or isotype control (Ig) antibody. Histological scoring of hematoxylin and eosin stained lung sections expressed as mean values ± SEM from five mice per group is shown.
- **J.** Histological assessment of mucus secretion in IL-28A (IL-28A) or vehicle control (PBS) treated OVA challenged wild-type mice in the presence of anti-IL-12p40 (anti-IL-12) or isotype control (Ig) antibody. Morphometric analysis of PAS stained sections expressed as mean values ± SEM from five mice per group are shown.
- **K.** T cell responses in MLNs of IL-28A (IL-28A) or vehicle control (PBS) treated OVA challenged wild-type mice in the presence of anti-IL-12p40 (anti-IL-12) or isotype control (Ig) antibody. Cytokine levels expressed as mean values ± SEM of five mice per group are shown. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, non-significant.

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well as spleen CD11c$^+$ DCs (Ank et al, 2008), express high levels of IL-28Rα and respond to IL-28A treatment to up-regulate known IL-28 target genes such as OAS1 and ISG15. More importantly, CD11c$^+$ lung or bone marrow derived DCs respond to IL-28A treatment to acquire a Th1-promoting phenotype. They up-regulate IL-12, a dominant Th1 cytokine that directly inhibits Th2 responses and promotes the production of IFN-γ. They also increase T-bet, an important transcription factor for promoting a Th1-inducing phenotype in DCs (Lugo-Villarino et al, 2003; Wang et al, 2006) and down-regulate OX40L, a key costimulatory molecule required for optimal induction of Th2 responses (Jenkins et al, 2007), although the rate-limiting role of these effects in IL-28A-mediated suppression is unclear.

Consistent with a critical role of DC modulation in IL-28-mediated suppression, immunization of naïve mice with IL-28A-treated DC promoted the induction of Th1 over Th2 or Th17 cell differentiation *in vivo* whereas adoptive transfer of CD11c$^+$ cells from IL-28A-treated mice (Supporting Information Fig S7) to naïve mice prior to OVA/alum immunization suppressed allergen sensitization and subsequent development of allergic disease. Moreover, administration of IL-28A or adoptive transfer of CD11c$^+$ cells from IL-28A-treated mice before inhaled allergen challenge inhibited effector Th2 cell responses in the lung and ensuing allergic airway inflammation. This was largely due to modulation of conventional CD11c$^+$MHCII$^+$CD11b$^+$ DC function, a major DC subset in the lung specialized for promoting primary and effector Th2 responses in the airways (Kool et al, 2008; Medoff et al, 2009; van Rijt et al, 2005). Although other DC subsets may also be involved in IL-28A-mediated Th1 skewing or suppression of allergic airway disease, our findings place modulation of CD11c$^+$MHCII$^+$CD11b$^+$ conventional DC function at the centre stage of IL-28A mode of action.

In addition to its role in DC modulation, IL-28A may also affect the activation state of the respiratory epithelium. IL-28Rα is expressed in the respiratory epithelium and IL-28A treatment reduces the expression of epithelium-derived cytokines such as IL-25 and TSLP involved in driving Th2 responses in the lung (Supporting Information Fig S8). However, whether this is due to direct effects on epithelial cells or indirect effects on the Th2 response which activates the respiratory epithelium remains to be determined.

An important role of IL-28 cytokines in T helper cell differentiation has been suspected. Transgenic expression of IL-28A was recently shown to augment the induction of Th1 responses and the severity of ConA-induced liver injury (Siebler et al, 2007). Moreover, addition of recombinant IL-29 was shown to inhibit Th2 cytokine production, especially IL-13, in human cell culture systems involving mitogenic stimulation, mixed-lymphocyte reaction and T cell differentiation assays (Dai et al, 2009; Jordan et al, 2007; Srinivas et al, 2008). This was associated with a modest increase in IL-12 production after LPS stimulation of monocyte-derived DCs and a tendency for increased IFN-γ production (Jordan et al, 2007), although direct inhibitory effects of IL-29 in GATA-3 expression of CD4$^+$ T cells and Th2 differentiation were also proposed (Dai et al, 2009). More recently, the possibility that IL-29 affects Th2 responses through the modulation of pDC function has also been raised (Megjugorac et al, 2009). Our study now builds up on these earlier observations. It provides the first *in vivo* evidence that IL-28A is critically involved in driving both primary and secondary Th1 responses at the expense of Th2 and Th17 cell differentiation while it reveals for the first time a role for endogenous IL-28 cytokines in this process. Modulation of conventional DC function to induce IL-12 and drive Th1 differentiation rather than the direct regulation of T cell function is central to IL-28A mediated suppression, raising the possibility that differences in this aspect between mice and humans may exist.

In summary, our study reveals a key regulatory role of IL-28A in allergic airway disease and provides new insight into the function of IL-28 cytokines in adaptive immunity. Our findings are consistent with a model in which low expression of IL-28 family cytokines during allergic inflammation and/or viral infection predisposes asthmatic patients for exacerbation of allergic airway disease both via reduced viral clearance and via inefficient suppression of Th2 and/or Th17 responses. As asthma constitutes a major 'epidemic' of our times, and as viral exacerbations of asthma are poorly controlled by current medication and account for the majority of hospitalizations of asthmatic patients (Sykes & Johnston, 2008), our findings advocate the clinical development of IL-28 cytokines for therapy.

MATERIALS AND METHODS

Cytokines, antibodies and recombinant adenoviruses

Cytokines and antibodies were from eBioscience (San Diego, CA), Peprotech (Rocky Hill, NJ) or BD (PharMingen, Heidelberg, Germany) as detailed in the Supporting Information Materials and methods section. LPS was from Sigma. Neutralizing anti-IL-12 p40 antibody (Clone C17.8) and its isotype control (Clone C1.18.4) were purchased from Bio-X-Cell (West Lebanon, USA). Recombinant replication-deficient E1/E3-deleted adenoviral vectors were constructed as described in the Supporting Information Materials and Methods section.

Allergen sensitization and challenge protocol and treatment regimens

Wild-type mice, IFN-$\gamma^{-/-}$ and IL-28R$\alpha^{-/-}$ mice on a C57BL/6J genetic background were maintained as previously described (Hausding et al, 2004). IL-28R$\alpha^{-/-}$ knockout mice were described elsewhere (Ank et al, 2008). In some experiments, BALB/cJ mice were also used. Unless indicated otherwise in Results section, 8–10 week old mice were sensitized with two intraperitoneal injections on days 0 and 7 of 7.5 μg OVA (Grade V; Sigma) complexed with aluminium hydroxide (alum) as adjuvant (Sigma, Deisenhofen, Germany). On days 14, 15 and 16, mice received aerosol challenge with 5% w/v OVA in PBS (OVA/OVA) for 30 min per day. Control mice received intraperitoneal injections and challenges of PBS alone (PBS/PBS). In some cases, mice were treated with 1 μg/mouse recombinant IL-28A concurrently with OVA challenges or 5×10^8 i.u. of an adenovirus expressing IL-28A (AdIL-28) and its mock control (Ad0) 1 day before allergen challenge as indicated in the experimental protocol. For adoptive transfer experiments, CD11c$^+$ DCs from AdIL-28, Ad0 or vehicle control (PBS)-treated mice were administered to mice 1 day before allergen challenge as detailed in the Supporting Information Materials and methods section. For IL-12

The paper explained

PROBLEM:

IL-28 cytokines or type III interferons exhibit potent antiviral and anticancer function. However, their full spectrum of activities in the immune system and their role in the context of an inflammatory disease remain poorly understood. This is despite recent evidence linking low levels of IL-28 cytokines with the severity of inflammation in asthma and viral exacerbations of asthma.

RESULTS:

Using IL-28R$\alpha^{-/-}$ mice, IL-28A gene transfer, recombinant IL-28A administration and an established model of experimental asthma in mice, we reveal a key function of IL-28 cytokines in inducing type 1 immunity and protecting from allergic airway disease. Abrogation of endogenous IL-28 function in IL-28R$\alpha^{-/-}$ mice augmented Th2 and Th17 responses as well as IgE levels and exacerbated allergic airway inflammation. In contrast, IL-28A treatment promoted Th1 responses, suppressed Th2 and Th17 responses and ameliorated allergic airway disease. This was due to IL-28A-mediated modulation of conventional DC function inducing IL-12p70 production and Th1 differentiation.

IMPACT:

This work extends our current knowledge on the mechanisms regulating allergic responses in the airways, provides new insight about the role of IL-28 cytokines in adaptive immunity and strengthens the rationale for the therapeutic administration of IL-28A in allergic asthma and related allergic diseases in humans.

p40 neutralization experiments, 300 μg/mouse of anti-IL-12p40 antibody or its isotype control were administered i.p. just before each treatment with recombinant IL-28A. One day after the last challenge mice were examined for AHR, bronchoalveolar lavage performed and tissues (MLNs, spleen and lung) collected for further analysis.

Airway hyper-responsiveness

Airway hyper-responsiveness was measured in anesthetized mechanically ventilated mice (flexiVent, SciReq, Montreal, Canada) 24 h after the last aerosol exposure as previously described (Xirakia et al, 2010). In BALB/c mice, AHR was measured invasively using a body plethysmograph (Buxco Electronics, Inc., Wilmington, NC) as previously reported (Hausding et al, 2007).

Differential cell counts, T cell responses and flow cytometric analysis

Differential cell counts of BALF cells, assessment of T cell responses in MLNs and lung, flow cytometric analysis, sorting and functional analysis of lung CD11c^{+} DCs were all performed as described in the Supporting Information Materials and methods section.

Histology and immunohistochemistry

Histopathologic analysis of inflammatory cells in H&E stained lung sections, morphometric analysis of PAS stained sections and immunohistochemistry were performed as described in the Supporting Information Materials and methods section.

Primary T cell responses and BM DC studies

Analysis of primary T cell responses and BM DC function were performed as described in the Supporting Information Materials and methods section.

Real time–PCR and ELISA

Real-time qRT-PCR and ELISA were performed as described in the Supporting Information Materials and methods section. For the analysis of IL-28 levels, BALF was concentrated 10X using Amicon Ultra-15 centrifugal filter columns (Millipore, USA) with a molecular weight cut-off of 10 kDa according to the manufacturer's instructions.

Statistical analysis

Differences were evaluated for significance ($p < 0.05$) by the Student's two-tailed *t*-test for parametric data and the Mann–Whitney U test for non-parametric data (Excel, PC). Data are given as mean values ± SEM.

Author contributions

EA and SF conceived and designed the experiments; OK, MH, AS, SK, CU and EA performed the experiments; EA, SF, MH, GT, HAL, MT, PS and MFN analysed the data; SVK, KMK and SED contributed reagents or analysis tools; EA and SF wrote the paper.

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Supporting Information is available at EMBO Molecular Medicine online.

Conflict of interest statement: Two of the authors, Kevin M. Klucher and Sean E. Doyle, are employees and stock holders

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of ZymoGenetics that owns intellectual property on IL-28 and therefore declare conflict of interest. The rest of the authors have no competing commercial interests in relation to the submitted work.

For more information

For general information about allergic asthma and allergic diseases:
European Academy of Allergy and Clinical Immunology (EAACI)
http://www.eaaci.net/

European Federation of Allergy and Airways Diseases Patients Associations (EFA)
http://www.efanet.org/

For related EU-funded research on allergy and asthma:
Global Allergy and Asthma European Network (GA2LEN)
http://www.ga2len.net/

Post-infectious immune reprogramming and its association with persistence and chronicity of respiratory allergic diseases
http://www.predicta.eu/

Webpage of the working group of E. Andreakos:
http://www.immunology-brfaa.gr/ and http://www.bioacademy.gr/

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