

Thymic stromal lymphopoietin plays divergent roles in murine models of atopic and nonatopic airway inflammation

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Abstract

Background: Thymic stromal lymphopoietin (TSLP) is a cytokine primarily produced by epithelial cells, which has been shown to be a potent inducer of T-helper 2 (Th2)-type responses. However, TSLP has pleiotropic effects upon immune cells, and although extensively studied in the context of atopic asthma, its relevance as a therapeutic target and its role in the pathogenesis of nonatopic asthma remains unknown. We sought to investigate the role of TSLP in atopic, nonatopic and viral-induced exacerbations of pulmonary inflammation.

Methods: Using stringently defined murine models of atopic, nonatopic and virally exacerbated forms of pulmonary inflammation, we compared inflammatory responses of C57BL/6 wild-type (WT) and TSLP receptor-deficient (TSLPR KO) mice.

Results: Thymic stromal lymphopoietin receptor (TSLPR) signaling was crucial for the development of atopic asthma. Specifically, TSLPR signaling to lung recruited CD4⁺ T cells enhanced eosinophilia, goblet cell hyperplasia, and overall inflammation within the airways. In contrast, the absence of TSLPR signaling was associated with strikingly exaggerated pulmonary neutrophilic inflammation in a nonatopic model of airway inflammation. The inflammation was associated with excessive levels of interleukin (IL)-17A in the lungs, indicating that TSLP negatively regulates IL-17A. In addition, in a model of influenza-induced exacerbation of atopic airway inflammation, the absence of TSLPR signaling also led to exaggerated neutrophilic inflammation.

Conclusion: Thymic stromal lymphopoietin plays divergent roles in the pathogenesis of atopic and nonatopic asthma phenotypes by either enhancing Th2 responses or curtailing T-helper 17 responses. These findings raise important caveats for the design of therapeutic interventions targeting TSLP in asthma.

The lung epithelium is a key regulator of pulmonary responses. It functions not only as a barrier but can also actively produce numerous mediators such as Thymic stromal lymphopoietin (TSLP), which can either dampen or exacerbate ongoing inflammation. Thymic stromal lymphopoietin is an IL-7-like cytokine, which signals via a heterodimeric complex comprising of the IL-7 receptor alpha (IL-7R α) and thymic stromal lymphopoietin receptor (TSLPR) subunit expressed on a broad range of cell types (1). Several studies have linked TSLP with protective Th2 responses against helminth parasites (2, 3) as well as detrimental Th2 responses culminating in allergic disorders such as asthma and atopic dermatitis (4, 5). In humans, TSLP expression is increased in asthmatic airways (6, 7), and the

Abbreviations

AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage; DC, dendritic cell; Ova, ovalbumin; pDC, plasmacytoid dendritic cell; Th17, T-helper 17; Th1, T-helper 1; Th2, T-helper 2; TSLPR^{−/−}, TSLP receptor knockout or TSLPR deficient; TSLP, thymic stromal lymphopoietin; WT, wild type.

absence of TSLP signaling is protective in a mouse model of allergic airway inflammation (8). Thymic stromal lymphopoietin can enhance Th2 responses either directly by acting on CD4⁺ Th2 cells to promote proliferation (8, 9) or indirectly by influencing dendritic cell maturation and their ability to polarize T cells (10–12). However, depending on the context of inflammation, TSLP can have a pleiotropic effect on the immune system beyond enhancing Th2 responses. In a recent study of influenza A infection, we showed that TSLP can boost virus-specific pulmonary CD8⁺ T-cell responses by modulating dendritic cell activation (13). Comparatively, in a model of DSS-induced colitis, TSLP can have an immunoregulatory role by facilitating epithelial cell repair and dampening the T-helper 1 (Th1) and T-helper 17 (Th17), cytokine response (3, 14, 15). Although TSLP is a potent inducer of Th2 responses and appears to be a promising candidate target for allergic asthma, it is also important to consider the diverse impact it has on the immune system and the implications this bears for the pathogenesis of different asthma phenotypes.

Asthma is a complex, heterogeneous disease, and not all asthma cases are associated with Th2-driven eosinophilic inflammation (16). Some epidemiological studies indicate that allergic disease accounts for less than half of asthma cases (17, 18). Indeed, neutrophils and Th17 responses are associated with the pathogenesis of more severe forms of the disease (19). Notably, conventional steroid treatments are ineffective against these forms of the disease (19, 20). We sought to elucidate the role of TSLP in the pathogenesis of different forms of asthma, using mouse models which allow the direct comparison of immune mechanisms underlying atopic (eosinophil-dominated) and nonatopic (neutrophil-dominated) asthma. Utilizing TSLPR-deficient mice, we show that TSLPR signaling was absolutely required for the development of atopic airway inflammation. In contrast, the absence of TSLPR signaling was not beneficial in the nonatopic model, but detrimental, leading to exacerbated neutrophilia and increased IL-17A production. Furthermore, during an influenza-induced exacerbation of the atopic airway inflammation, TSLP also regulated neutrophilic inflammation. These data highlight the context-dependent regulatory or proinflammatory impact of TSLP in airway inflammation.

Materials and methods

Mice

C57BL/6 mice were purchased from Charles River, l'Arbresle, Cedex, France. TSLPR-deficient mice on a C57BL/6 background were bred locally in a specific pathogen free facility. Mice used were between 8 and 15 weeks old at the start of the experiment. Animals were age and sex matched for all experiments. All animal experiments were performed according to institutional guidelines and Swiss federal and cantonal laws on animal protection.

Ovalbumin-induced atopic and nonatopic airway inflammation

Previously published model for atopic and nonatopic airway inflammation (21) was modified in the study as described below.

Sensitization

For the atopic inflammation model, mice were immunized intraperitoneally with either phosphate buffered saline (PBS) or 50 µg of ovalbumin (OVA, grade V; Sigma-Aldrich, Saint Louis, MO, USA) in 200 µl alum adjuvant (SERVA Electrophoresis GmbH, Heidelberg, Germany) twice over 2 weeks.

For the model of influenza-induced exacerbation of atopic airway inflammation, 50 PFU of influenza A virus strain PR8 (A/Puerto Rico/8/34, H1N1; sourced from Virapur LLC, San Diego, CA) was administered together with the first challenge of ovalbumin.

For the nonatopic inflammation model, 50 µg of ovalbumin in PBS was emulsified in equal volume of complete Freund's adjuvant (CFA Chondrex Inc., Redmond, WA, USA) and administered subcutaneously in a volume of 200 µl, twice over 2 weeks on opposite flanks.

Antigen challenge

A week after the second immunization, the mice were subjected to intranasal challenges of either PBS or 150 µg of ovalbumin alone on four consecutive days. Analysis was performed 1 day after the final challenge.

Measurement of lung function

Lung resistance was measured using the whole-body-restrained plethysmograph system flexiVent from Scireq. Mice were anesthetized by intramuscular injection of 100 mg/kg ketamine (Ketasol-100; Dr. E. Graeb AG, Bern, Switzerland) and intraperitoneal injection of 50 mg/kg pentobarbital (Esconarkon; Streuli Pharma, Uznach, St Gallen, Switzerland). Subsequently, mice were tracheotomized and mechanically ventilated at a rate of 200 breaths/min and a tidal volume of 10 ml/kg bodyweight. Airway hyper-responsiveness (AHR) was measured by administering increasing doses of acetyl methyl choline (Sigma).

Adoptive transfer experiments

Mice were immunized intraperitoneally twice over 2 weeks with ovalbumin in alum adjuvant. Cells isolated from the spleens and mediastinal lymph nodes of immunized mice were further cultured with ovalbumin for 5 days. CD4⁺ T cells were isolated from cultured cells by magnetic separation (MACS; Milteny Biotech, Bergisch, Germany) prior to intravenous transfer into naïve recipient animals. The recipients were subsequently challenged with ovalbumin over 3 days. Airway eosinophilia was assessed after the last challenge.

Neutralizing IL-17A during antigen challenge

Neutralizing anti-IL-17A (17-F3) antibody and corresponding isotype control mouse IgG1 (MOPC-21) antibody were purchased from bioXcell, West Lebanon, NH, USA. 250 µg of either IL-17A or isotype antibody was administered starting 1 day prior to antigen challenge and following that every 2 days until the final outcome.

Statistical analysis

Unpaired, two-tailed Student's *t*-test (Prism version 5 software; GraphPad, La Jolla, CA, USA) was used to calculate significance levels between treatment groups. *P* values of <0.05 were considered significant.

Results

TSLP is critical for the development of atopic airway inflammation

Using an ovalbumin-induced model of atopic airway inflammation (Fig. 1A), we compared responses between wild-type (WT) and TSLPR knockout (TSLPR^{-/-}) mice. In line with

previous studies with this model (8), we found that TSLPR-deficient mice exhibited an impaired inflammatory response characterized by a marked reduction in goblet cell hyperplasia (Fig. 1B) and cellular infiltration in the airways (Fig. 1C). Thymic stromal lymphopoietin receptor deficiency resulted in a dampened recruitment of eosinophils (Fig. 1D) and reduced airway hyper-responsiveness (AHR; Fig. 1E). Moreover, in the TSLPR^{-/-} mice, there was a defect in the overall magnitude of Th2 response as demonstrated by reduced levels of IL-4, IL-5, and IL-13 in the lung (Fig. 1F), as well as IgE antibody levels in the bronchoalveolar lavage (BAL) fluid (Fig. 1G).

TSLP does not influence the priming or polarization of CD4⁺ T-cell responses in the draining lymph node

We assessed whether the impaired atopic airway inflammation in TSLPR^{-/-} mice was a consequence of a defect in the initiation of the Th2 response in the draining lymph node. We did not observe a reduction in the total number of cells in the lymph node of TSLPR^{-/-} mice (Fig. 2A). Moreover, cells from TSLPR^{-/-} draining lymph nodes proliferated with a similar capacity to the WT in response to ovalbumin (Fig. 2B) TSLPR deficiency did not alter the

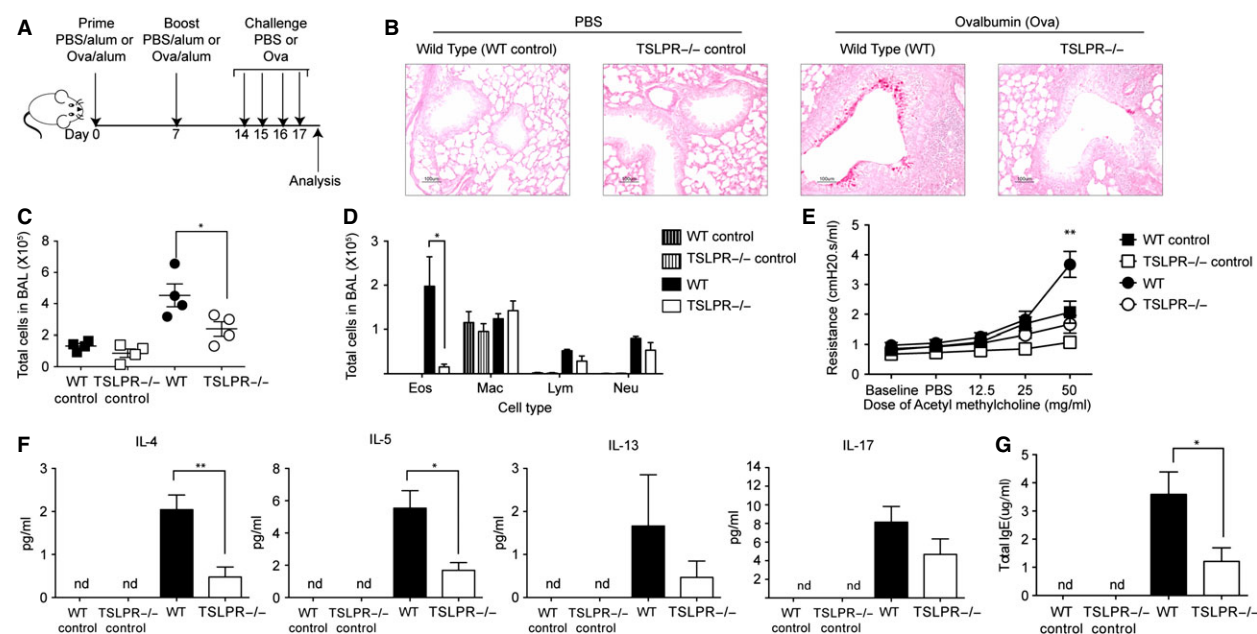


Figure 1 Thymic stromal lymphopoietin receptor (TSLPR) signaling is crucial for the development of atopic airway inflammation. (A) Wild-type and TSLPR-deficient mice were sensitized intraperitoneally with phosphate buffered saline (PBS) (control) or ovalbumin (Ova) in alum at day 0 and 7. They were subsequently challenged intranasally with PBS or Ova in PBS as indicated. Analysis was performed at day 4 after the first challenge. (B) Goblet cell hyperplasia and mucus production were determined by periodic acid–Schiff (PAS) staining of formalin-fixed lung sections. Images are representative of two independent experiments (*n* = 3–4). (C) Total cells infiltrating the bronchoalveolar lavage (BAL) were enumerated. (D) The number of

eosinophils, macrophages, lymphocytes, and neutrophils in the BAL was determined by Diff Quik staining. Data are representative of at least five independent experiments (*n* = 3–5). (E) Total lung resistance to incremental doses of acetyl methyl choline was measured using a whole-body-restrained plethysmograph (for test groups *n* = 6–8, for control groups, *n* = 3 data pooled from two experiments). (F) Cytokine protein levels were measured in lung homogenate using a milliplex assay kit. Levels were normalized to total protein levels per milligram of lung homogenate (*n* = 8–7, data pooled from two experiments). (G) Total IgE levels were quantified in the BAL fluid by ELISA (*n* = 5 per group). **P* = 0.05, ***P* = 0.01.

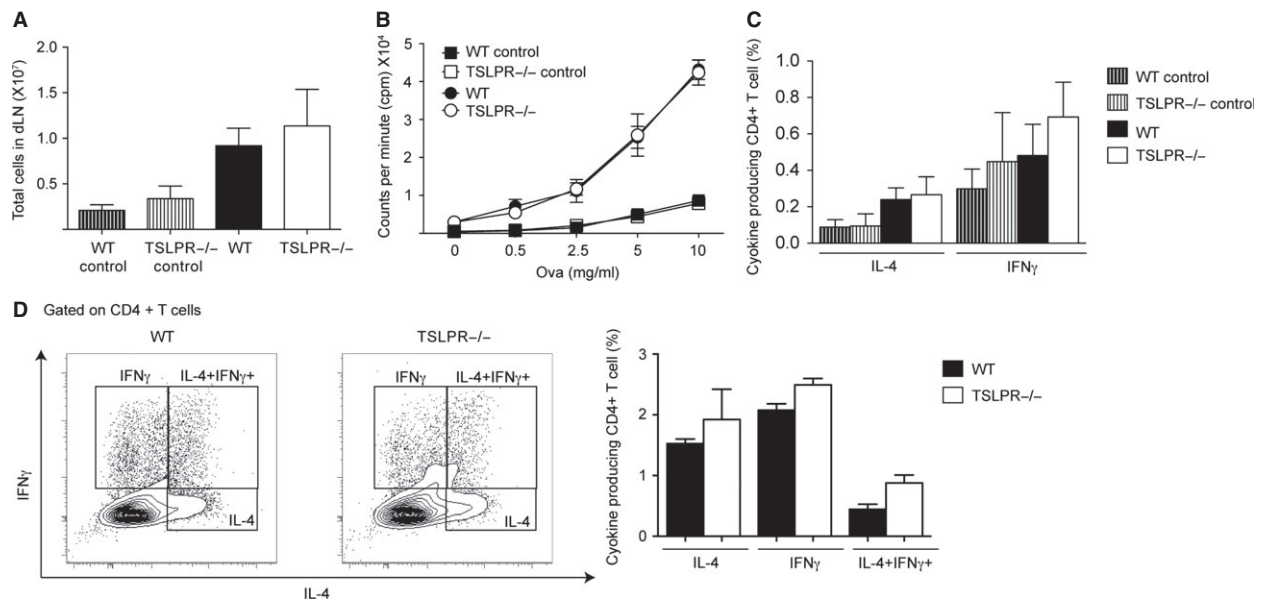


Figure 2 Thymic stromal lymphopoietin does not affect priming or polarization of CD4⁺ T-cell responses in the lung draining lymph node. (A) Total cell counts were determined in the mediastinal lymph node at day 4 after the last intranasal challenge. (B) Total lymph node cells were cultured in the presence of indicated concentrations of ovalbumin for 48 h. Thymidine incorporation in the last 12 h was measured as an indicator of cell proliferation. (C)

Cytokine production by CD4⁺ T cells in the draining lymph node was determined by flow cytometry ($n = 3-4$ per group). (D) Cells isolated from spleen and mediastinal lymph nodes of Ova/alum-primed mice were cultured *ex vivo* in the presence of Ova for 5 days. Phorbol-12-myristate-13-acetate ionomycin and brefeldin were added in the last 4 h, and cytokine production was determined by flow cytometry.

polarization of CD4⁺ T-cell responses in the draining lymph node as evaluated by the production of IL-4 and interferon gamma (IFN γ ; Fig. 2C). Moreover, following antigen-specific restimulation, cells isolated from the spleen and mediastinal lymph nodes of ovalbumin-sensitized mice showed a similar capacity for cytokine production (Fig. 2D). Overall, the priming of CD4⁺ T cells was unaffected by TSLPR deficiency.

TSLP acts directly on pulmonary CD4⁺ T cells to induce atopic airway inflammation

As the responses in the draining lymph node were unaffected, we considered that TSLP might act locally to amplify the atopic airway responses. Several studies have linked the action of TSLP on dendritic cells (DCs) to their ability to polarize Th2 responses (10–12). We characterized the DC subsets (Fig. S1) and their activation in the lungs. We observed a decrease in the number of CD11b⁺ DCs and plasmacytoid DCs (pDCs) in the lungs of TSLPR^{-/-} mice (Fig. 3A). However, their activation status as evaluated by the expression of CD40, ICOSL, and CD80 was unaltered in the absence of TSLPR signaling (Fig. 3B). Fewer numbers of these DC subsets in the lungs of TSLPR^{-/-} mice may be indicative of reduced inflammation as opposed to a cause of the defective Th2 response.

It has been reported that TSLP can act directly on CD4⁺ T cells and induce their proliferation (22, 23). In line with this, we also observed that TSLPR^{-/-} mice exhibited

reduced numbers of CD4⁺ T cells in the airways (Fig. 3C). To address whether TSLP targets CD4⁺ T cells directly during the disease, we performed adoptive transfer experiments. Splenic CD4⁺ T cells from Ova/alum-immunized mice were expanded with ovalbumin *in vitro* and transferred into naïve recipients (Fig. 3D). The recipients were then challenged with ovalbumin intranasally, and the recruitment of eosinophils to the airways was assessed. We observed a decrease in the cell infiltration into the BAL (Fig. 3D) and a specific defect in eosinophilia only in the mice that received CD4⁺ T cells from TSLPR^{-/-} donors (Figs 3E and S2). Additionally, we observed a reduction in the number of total donor CD4⁺ T cells recovered in these two compartments when cells from TSLPR^{-/-} donor mice were transferred (Fig. 3F) although their cytokine production was comparable (Fig. 3G, H). Therefore, in our model, TSLP plays a role in driving atopic airway inflammation by directly targeting CD4⁺ T cells.

TSLP is not required for the development of nonatopic airway inflammation

As TSLP is critical in the development of atopic airway inflammation, we sought to investigate whether it could also play a role in nonatopic forms of the disease. To model nonatopic asthma, we immunized mice subcutaneously with ovalbumin emulsified in CFA (Fig. 4A) as this biases the immune response toward Th1 and Th17 with predominantly neutrophilic inflammation, mimicking the more severe forms of nonatopic asthma.

In contrast to the atopic airway inflammation model, TSLPR^{-/-} mice exhibited a more severe inflammatory response in the nonatopic model. The lungs of TSLPR^{-/-} mice showed an increased cellular infiltration (Fig. 4B). Similarly, we also observed an increase in the cellular influx (Fig. 4C) dominated by neutrophils (Fig. 4D) in the airways of TSLPR^{-/-} mice. The most striking difference we measured was, however, the dramatic increase in baseline resistance in the TSLPR^{-/-} mice which is reflective of severe disease (Fig. 4E).

Furthermore, although IFN γ production was similar between WT and TSLPR^{-/-}, we observed a marked increase in the total level of IL-17A expression in the lungs as well as the proportion of IL-17A-producing CD4⁺ T cells in the BAL of TSLPR^{-/-} mice (Fig. 4F, G). Thus, while TSLP amplified Th2-driven inflammation in the atopic model, it alleviated the inflammation in the nonatopic model.

IL-17A plays a key role in the pathogenesis of nonatopic airway inflammation

To directly assess the contribution of IL-17A in the pathogenesis of nonatopic airway inflammation, we administered neutralizing IL-17A antibody every 2 days starting from 1 day before the first intranasal challenge with ovalbumin (Fig. 5A). While neutralizing IL-17A did not have a significant impact on the inflammation in the lung parenchyma (Fig. 5B), it resulted in a reduction of the total cellular influx into the airways (Fig. 5C) most notably affecting neutrophils (Fig. 5D). It also ameliorated airway hyper-responsiveness (Fig. 5E). We also neutralized IL-17A in the atopic airway inflammation model, and although this did not influence Th2-driven responses such as eosinophilia and IgE, we observed that neutrophil influx was decreased in line with data from the nonatopic disease (Fig. S3). Taken together, these data show that IL-17A plays a key role in driving airway neutrophilia during atopic and nonatopic airway inflammation.

TSLP regulates neutrophilic inflammation in influenza-induced exacerbation of atopic airway inflammation

Respiratory viral infections are a common trigger for exacerbations of asthma. Of note, we observed that TSLPR^{-/-}

mice exhibited an exaggerated neutrophilic response during influenza infection (Fig. S4). We thus investigated whether TSLP could play a similar role in virus-induced exacerbations of atopic airway inflammation. To model virus-induced exacerbation of atopic asthma, we administered a low dose of influenza A virus at the time of the first ovalbumin challenge (Fig. 6A). We found that goblet cell hyperplasia (Fig. 6B) was reduced in TSLPR-deficient mice, irrespective of the viral infection; however, TSLPR-deficient mice exhibited an overall increase in cellular infiltration in the BAL (Fig. 6C), which was attributed to an increased infiltration by neutrophils (Fig. 6D). In comparison with the standard model of atopic airway inflammation, viral-induced exacerbation of the atopic response increased airway hyper-responsiveness in the TSLPR^{-/-} mice (Fig. 6E), while Th2 cytokines remained reduced (Fig. 6F–H). These data suggest that the blockade of TSLP during viral exacerbations could potentially serve to further exacerbate the response.

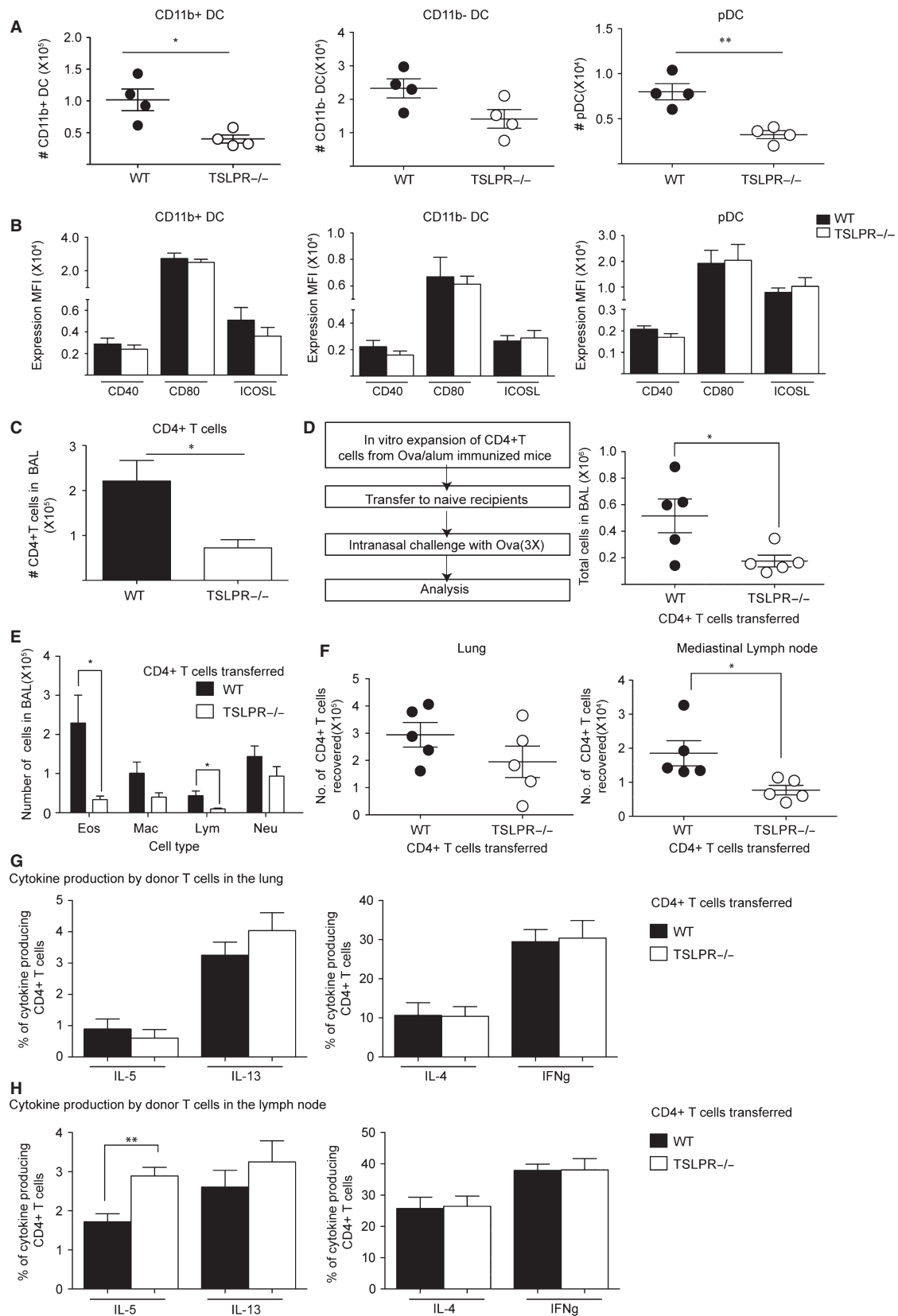
Discussion

Thymic stromal lymphopoietin has been attributed a key role in the pathogenesis of allergic airway disease (5, 24). It can promote Th2 responses by acting on dendritic cells, innate immune cells, and CD4⁺ T cells (8–12). In agreement with a previous study using a similar model of atopic airway inflammation (8), we find that TSLP enhances the disease by directly acting on CD4⁺ T cells in the lung. However, a recent study using house dust mite allergen showed that TSLP played an important role in pathogenesis only when a higher dose of allergen challenge was used (25). In humans, although TSLP expression can fluctuate in asthmatics, it positively correlates with Th2 inflammation (6, 7). Collectively, these data suggest that therapeutic interventions targeting TSLP are more likely to be efficacious in severe eosinophil-dominated forms of atopic asthma.

In addition to its role in Th2 responses, TSLP can influence other inflammatory pathways. For instance, during primary infection with the virus, influenza A, TSLPR-deficient mice exhibit an enhanced neutrophilic response at the peak of viral replication (Fig. S4). Neutrophils also predominate in acute exacerbations of the disease (26), which can be

Figure 3 Thymic stromal lymphopoietin does not affect pulmonary dendritic cell maturation but acts directly on CD4⁺ T cells to induce atopic airway inflammation. (A) At day 4 after the last challenge, the total number of CD11b⁺, CD11b⁻, and plasmacytoid dendritic cells (pDCs) was determined by flow cytometry. Data are representative of two independent experiments ($n = 3$ –4). (B) Activation of dendritic cells was measured by quantifying the expression of CD40, CD80, and ICOSL ($n = 7$ –8, data pooled from two experiments). (C) Total number of CD4⁺ T cells in the BAL was quantified by flow cytometry ($n = 5$). (D) Wild-type and thymic stromal lymphopoietin receptor (TSLPR^{-/-}) donors were immunized twice with Ova (d0 and d7) before collection of the spleens and lymph nodes on day 14. Splenocytes and cells from mediastinal lymph

node of Ova-primed animals were cultured *in vitro* with Ova for an additional 5 days prior to i.v. transfer into naïve congenic wild-type recipient animals. Naïve recipients were then challenged with Ova intranasally on three consecutive days. Total cells infiltrating the bronchoalveolar lavage (BAL) were enumerated. (E) The number of eosinophils, macrophages, lymphocytes, and neutrophils in the BAL was determined by Diff Quik staining. (F) The total number of donor CD4 recovered in lung and mediastinal lymph node of recipient mice was determined using congenic markers by flow cytometry. (G) Cytokine production by donor CD4⁺ T cells in the lung and (H) draining lymph node was determined by flow cytometry after *ex vivo* stimulation with PMA ionomycin and brefeldin for 5 h. * $P = 0.05$, ** $P = 0.01$.



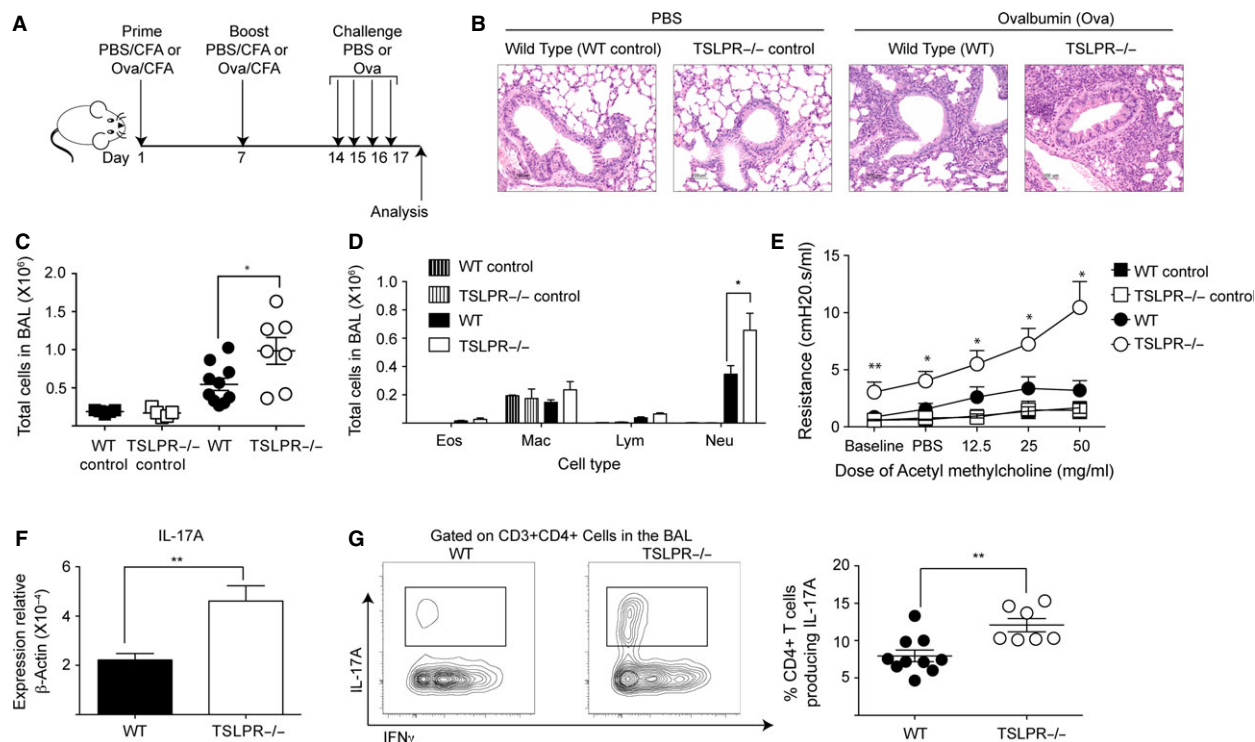


Figure 4 Thymic stromal lymphopoietin is not required for the development of nonatopic airway inflammation. (A) Wild-type and thymic stromal lymphopoietin receptor (TSLPR)-deficient mice were sensitized subcutaneously with Ova in complete Freund's adjuvant (CFA) at day 0 and 7 in opposing flanks. They were subsequently challenged intranasally with Ova in phosphate-buffered saline as indicated. Analysis was performed at day 4 after the first challenge. (B) Representative picture of H&E-stained formalin-fixed lung section. (C) Total cells infiltrating the BAL were enumerated. Data are pooled from two experiments. (D) The total number of eosinophils, macro-

phages, lymphocytes, and neutrophils in the BAL was determined following Diff Quik staining. (E) Total lung resistance to incremental doses of acetyl methyl choline was measured using the whole-body-restrained plethysmograph system flexiVent from Scireq (for test groups $n = 7-8$, for control groups $n = 2-4$, data pooled from two experiments). (F) IL-17A expression in the lungs of Ova/CFA-immunized and Ova-challenged mice was quantified by real-time PCR ($n = 9-7$ data pooled from 2 experiments). (G) Cytokine production by CD4+ T cells in the lung was determined by flow cytometry. Data are pooled from two experiments. * $P = 0.05$, ** $P = 0.01$.

triggered by viral infections (27). We found that TSLP negatively regulated neutrophilic inflammation in an influenza-induced exacerbation of atopic airway inflammation. These data raise important caveats in targeting TSLP during exacerbations of atopic asthma.

Next, we investigated the pathways through which TSLP could negatively regulate neutrophil responses. In models of bacterial and viral exacerbations of atopic airway inflammation, neutrophilic responses are mediated by IL-17A (28, 29). Interestingly, in the intestine, commensal-driven homeostatic TSLP expression regulates the production of proinflammatory cytokines including IL-17A (30). In models of colitis, TSLP can alleviate inflammation either by regulating IL-17A levels or by facilitating epithelial cell repair (3, 14, 15). Although we did not monitor the kinetics of IL-17A production following infection, at the peak of viral replication we did not observe any difference in the expression of IL-17A in WT and TSLPR $^{-/-}$ mice. Moreover, in the absence of exacerbation, we observed that TSLPR-deficient mice exhibited reduced disease, while levels of IL-17A were unaffected (Fig. 1F). Thus, although IL-17A can play a role in driving

inflammation, it is not regulated by TSLP in atopic airway inflammation.

Although the exact role of IL-17A in murine models of atopic asthma is controversial, it has been shown that it is involved in the development of asthma in the sensitization phase (31, 32). In contrast, neutralizing IL-17A during allergen challenge inhibits AHR (33). As compared to this previous study (33), we found that neutralizing IL-17A during the effector phase did not enhance the inflammation or cellular influx into the airways and resulted in only a minor increase in eosinophilia but a significant decrease in the proportion of neutrophils and lymphocytes (Fig. S3).

Considering the impact of TSLP in neutrophilic responses during exacerbation of atopic asthma, we speculated whether TSLP might play a regulatory role in a Th1-/Th17-driven model of nonatopic airway inflammation. Indeed, we observed that TSLPR deficiency amplified the ongoing inflammation, and this correlated with increased neutrophil influx and increased levels of IL-17A. In line with the data from a colitis model (3, 15), TSLP also appears to limit IL-17A release in the lungs during neutrophil-dominated

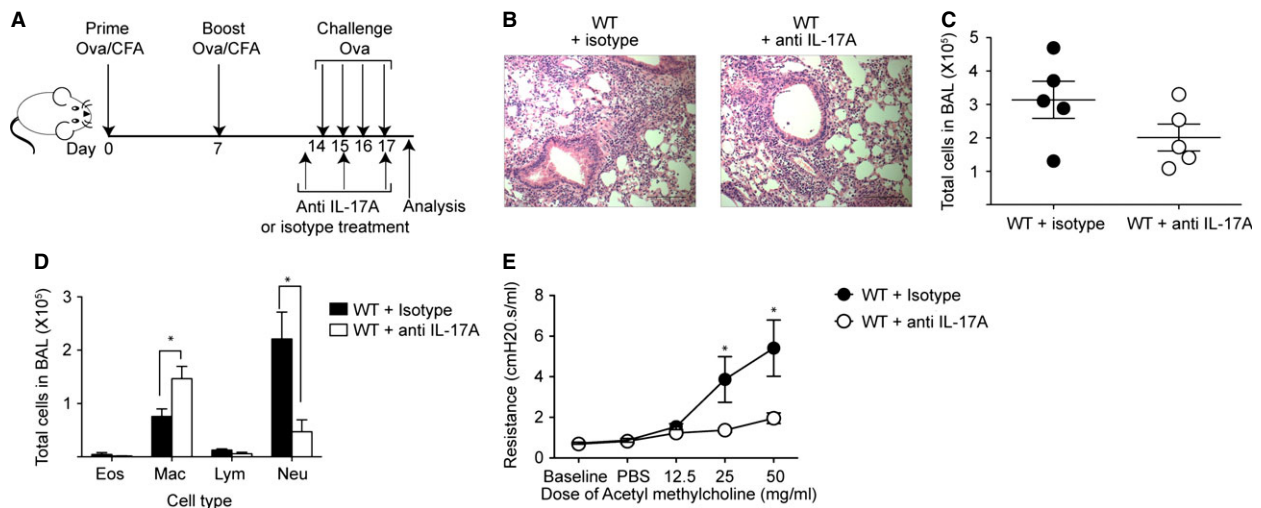


Figure 5 IL-17A plays a crucial role in enhancing nonatopic airway inflammation. (A) IL-17A was neutralized in wild-type mice at the indicated time points. (B) H&E-staining of formalin-fixed lung sections. (C) Total cells infiltrating the BAL were enumerated. (D) The total

number of eosinophils, macrophages, lymphocytes, and neutrophils in the BAL was determined following Diff Quik staining ($n = 5$ per strain). (E) Total lung resistance to incremental doses of acetyl methyl choline was measured ($n = 4-5$ per dose per group). * $P = 0.05$

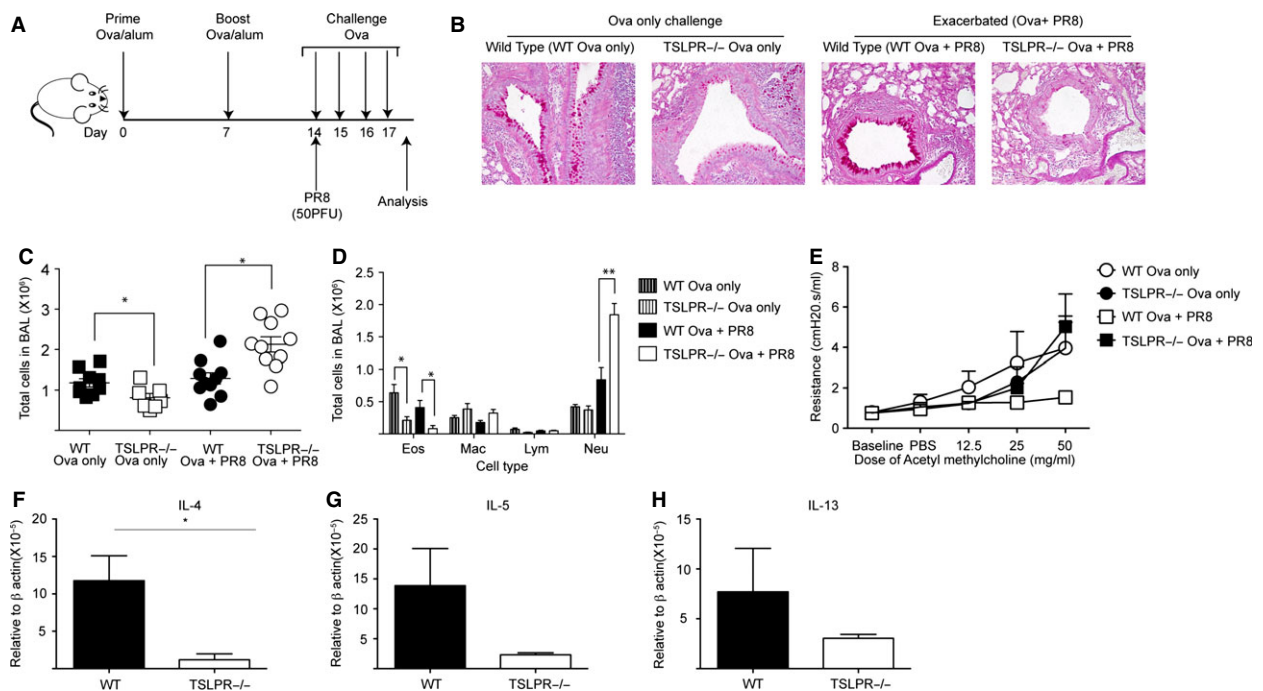


Figure 6 Thymic stromal lymphopoietin (TSLP) regulates neutrophilic inflammation in influenza-induced exacerbation of allergic airway inflammation. (A) Wild-type and thymic stromal lymphopoietin receptor (TSLPR)-deficient mice were sensitized intraperitoneally with ovalbumin (Ova) in alum at day 0 and 7. On day 14, they were challenged with Ova alone or 50 PFU of PR8 and Ova in PBS. This was followed by three additional challenges of Ova alone. Analysis was performed at day 4 after the first challenge. (B) Goblet cell hyperplasia and mucus production were determined by periodic acid-Schiff (PAS) staining of formalin-fixed lung sections. (C) Total

cells infiltrating the bronchoalveolar lavage (BAL) were enumerated (data pooled from 2 experiments) (D) The number of eosinophils, macrophages, lymphocytes, and neutrophils in the BAL was determined by Diff Quik staining ($n = 5$ per group). (E) Total lung resistance to incremental doses of acetyl methyl choline was measured using a whole-body-restrained plethysmograph ($n = 4-5$ per dose per group). (F-H) Cytokine mRNA expression was measured in lungs of mice challenged with Ova and PR8. Levels were normalized to beta-actin expression ($n = 3-4$). * $P = 0.05$, ** $P = 0.01$.

nonatopic pulmonary inflammation. Although IL-17A is increased in both atopic and nonatopic asthma (34–36), we found a more striking role for IL-17A in the pathogenesis of nonatopic airway inflammation. Neutralizing IL-17A resulted in a reduced infiltration of cells into the airways, specifically a marked reduction in the neutrophil recruitment and an alleviation of airway hyper-responsiveness.

The heterogeneity underlying the pathogenesis of different asthma phenotypes poses a serious problem in its treatment (19, 20). We demonstrate a dichotomy in the role of TSLP in atopic and nonatopic models of airway inflammation. In the atopic model, TSLP clearly plays an important role in driving Th2-mediated inflammation. In contrast, TSLP can counter-regulate the Th17 pathway and significantly reduce neutrophilic inflammation during acute exacerbations of atopic airway inflammation and in nonatopic airway inflammation. Taken together, our data highlight the importance of considering the diverse functions of cytokines *in vivo* for the development of novel therapeutics and treatment regimes in complex pulmonary diseases such as asthma.

Author contributions

Koshika Yadava, Joanna Massacand, and Ilaria Mosconi performed experiments and analyzed data. Koshika Yadava, Laurent P. Nicod, Nicola L. Harris, and Benjamin J.

Marsland conceived of the project and wrote the manuscript.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Gating strategy for dendritic cells in the lung.

Figure S2. TSLP acts directly on CD4+ T cells to drive atopic airway inflammation.

Figure S3. IL-17A influences neutrophilia in atopic airway inflammation.

Figure S4. TSLP regulates neutrophilia during infection with influenza.

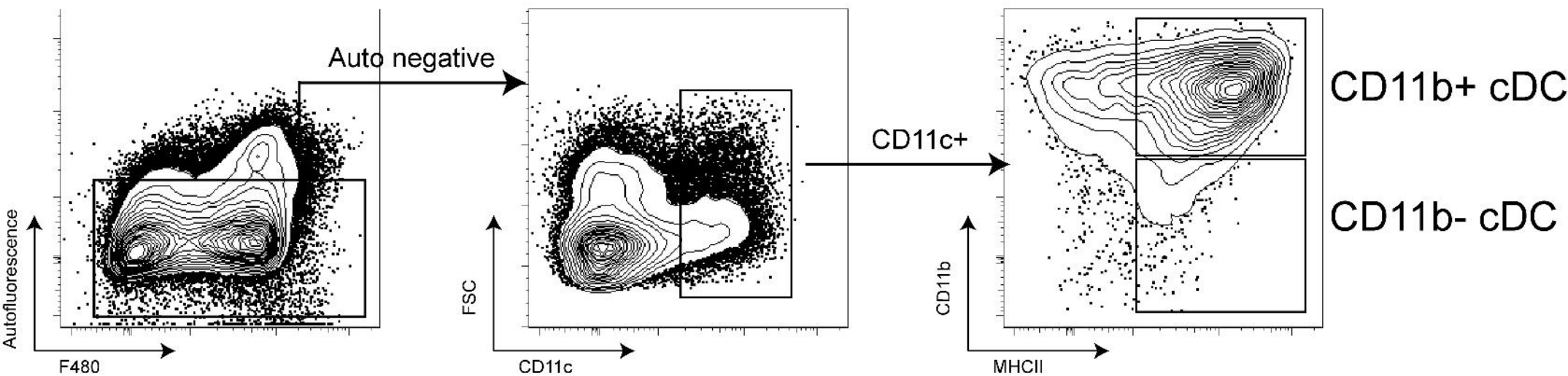
Appendix S1. Supplementary Methods.

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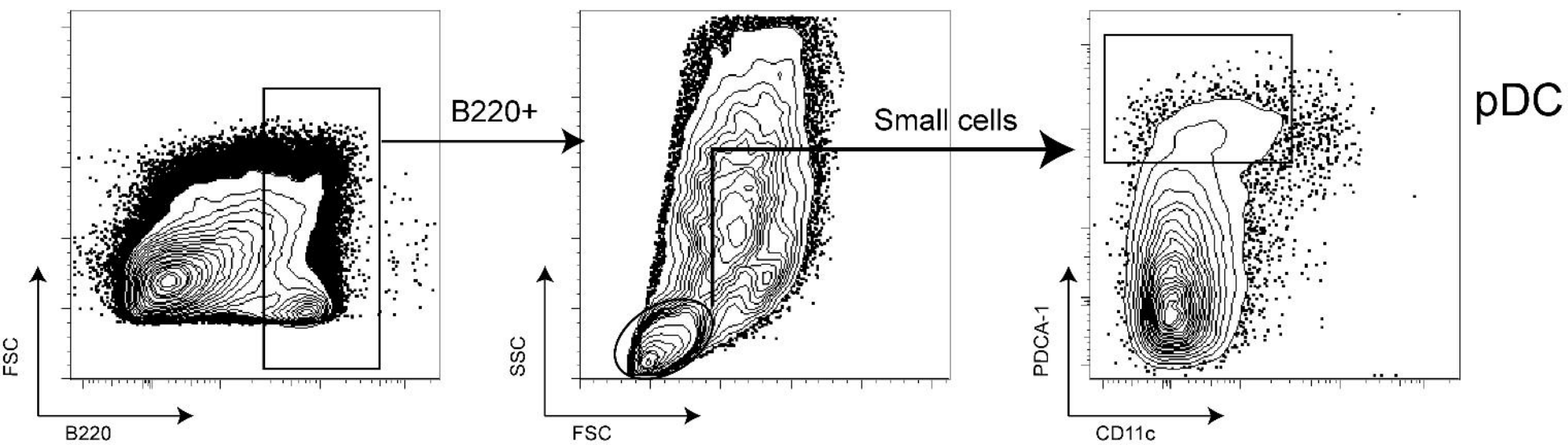
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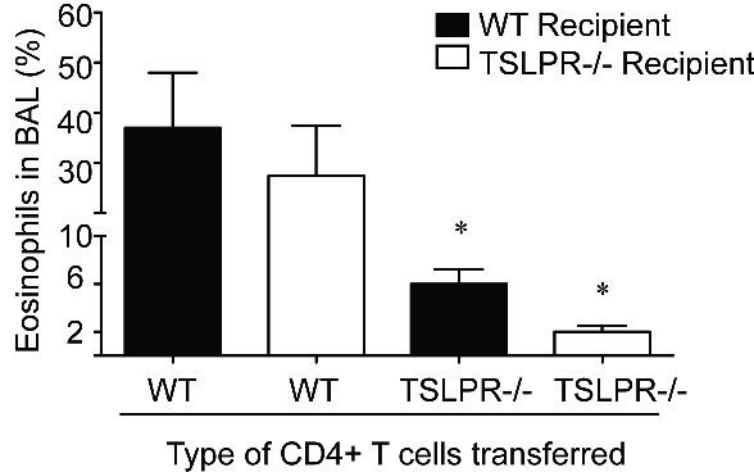
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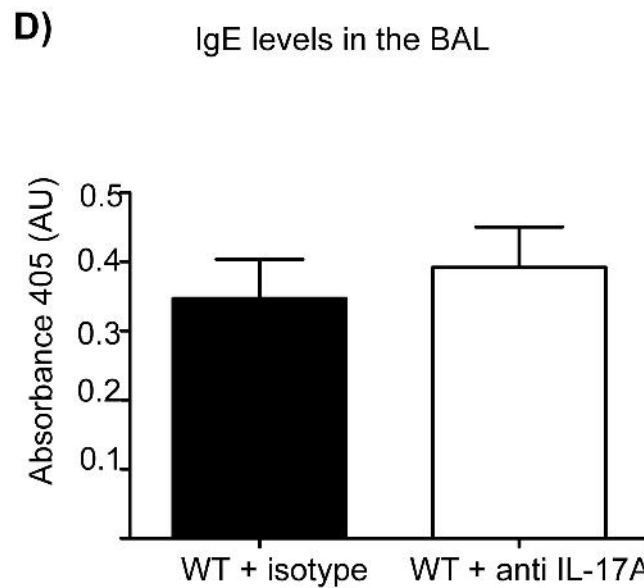
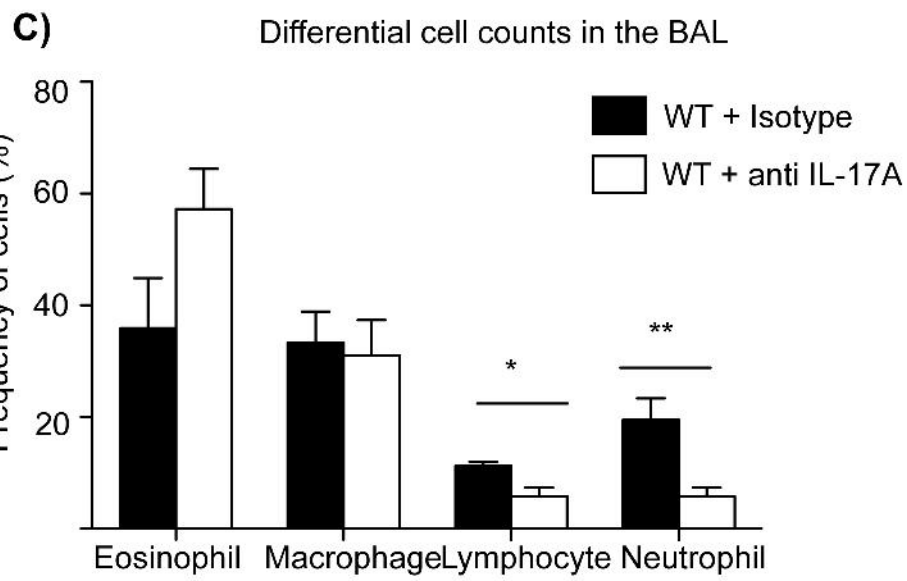
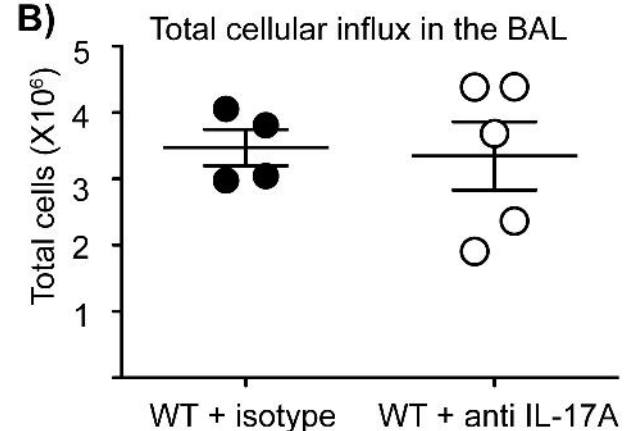
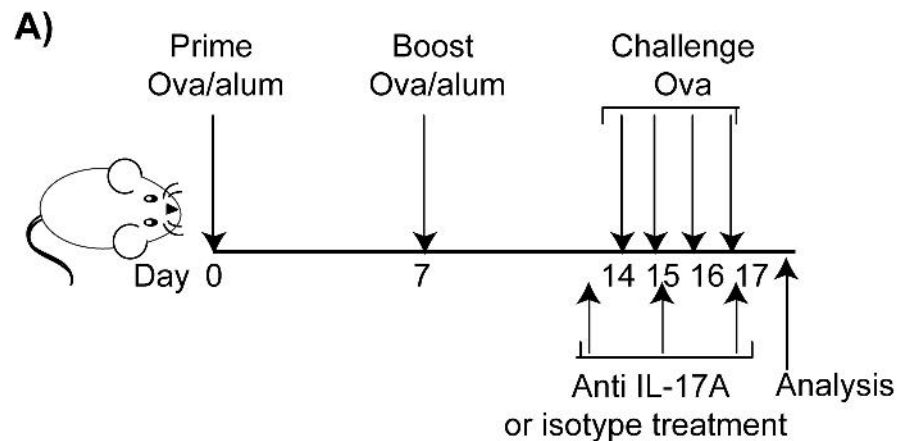
Gated on live, single cells



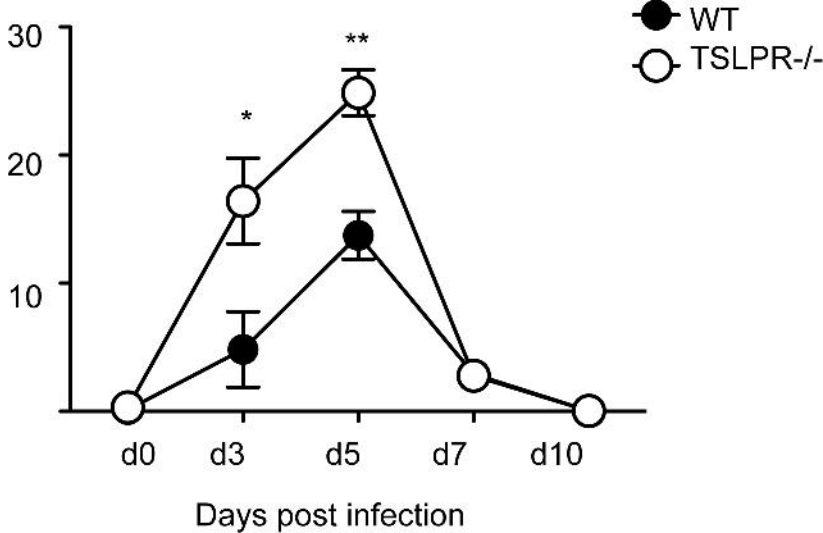
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Frequency of cells
in the BAL (%)



SUPPLEMENTARY METHODS

Analysis of cells from the bronchoalveolar lavage and lungs: BAL was performed by flushing the airways twice with 500 μ l of PBS/0.1% BSA. Total cell counts were determined using a Beckmann coulter counter. Cells from BAL were spun onto glass slides using a cytopsin3 Shandon. Slides were then stained using Diff Quick staining set (Dade Behring, Siemens Healthcare Diagnostics, Deerfield, IL) and differential cell counts were determined microscopically. percentages of eosinophils, neutrophils, macrophages, and lymphocytes were determined within a total population of 200 cells. For isolation of cells from the lung, lungs were perfused with PBS and then digested in medium supplemented with 2 mg/ml Collagenase IV (Invitrogen-gibco). Single cell suspension was obtained by smashing digested lungs through a 70 μ M cell strainer (Milian Falcon).

Histology Entire lungs or only the left lung lobe were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, USA) or 10% formalin, embedded into paraffin and stained with hematoxylin and eosin or Periodic acid Schiff reaction.

Cytokine production and cell proliferation assays: Single cell suspension was obtained from different tissues such as BAL, lung and lymph node. Cells were incubated with PMA (Sigma) ionomycin (FLUKA-Sigma) in the presence of monensin (FLUKA-Sigma) for 4 hours. Intracellular cytokine production was determined by flow cytometry as described below. For measuring cell proliferation, cells isolated from lung draining lymph nodes were cultured in the presence of different concentrations of ovalbumin for 72 h at 37°C with methyl [³H] thymidine, (1 mCi/well) (Perkin Elmer) added for the last 18 h, and total [3H] thymidine incorporation was measured.

Antibodies and flow cytometry: Different combinations of the following antibodies were used for flow cytometry. For analysis of dendritic cell subsets in the lung a combination of CD11b PerCP-Cy5.5, CD11c APC-Cy7, B220 FITC, Ly6c pacific blue, MHC II Alexa Fluor 700, PDCA-1 Alexa Fluor 647, CD40 PE, CD80 biotin, ICOS-L biotin and streptavidin PE-Cy7 was used. For the intracellular staining of restimulated cells a combination of CD3 pacific blue, CD4 PercpCy5.5, CD8a PE-Cy7, IFN γ FITC, IL-17A Alexa 700. All antibodies were purchased from BioLegend. Stained cells were acquired on a BD FACS Calibur or BD FACS CANTO or BD FACS LSR II and analyzed on FlowJo software (Tree star, Inc).

Antibody and cytokine measurements: Interleukin (IL-) 4, IL-5, IL-13, IL-17, and interferon (IFN) γ were measured in Lung homogenate using a Milliplex assay kit (Millipore, Billerica, MA). Assays were performed according to manufacturers' instructions and read on the Luminex 200™ Multiplexing Instrument (Merck Millipore). Values were normalized to total lung protein levels determined by Bradfords assay. The levels of total antibody isotypes IgE (Bioconcept, Southern Biotech) in the BAL fluid were determined by sandwich ELISA. Alkaline phosphatase conjugated secondary antibodies and p-nitrophenyl phosphate (Sigma) as substrate was used. OD was determined at 405 nm using a synergy H1 microplate reader (Biotek Instruments Inc.)

Quantitative real time PCR: Total RNA from lung tissue was purified using tri reagent (Molecular Research Centre Inc.) RNA was converted to cDNA using iscript cDNA synthesis kit (Biorad). cDNA was quantified using SSO advanced sybr green supermix (Bio-rad) real-time PCR by CFX96 real time system from Bio-Rad.

Expression of target genes was normalized to beta actin expression levels. The following primers were used IL-17A forward 5'-ACC CTG GAC TCT CCA CCG CAA-3', IL-17A reverse 5'-GGT GGT CCA GCT TTC CCT CCG-3' beta actin: forward 5'-CCC TGA AGT ACC CCA TTG AAC-3' beta actin reverse: 5'-CTT TTC ACG GTT GGC CTT AG-3', IL-4 forward 5'-TGT CAT CCT GCT CTT CTT TCT C-3', IL-4 reverse 5'-GCA CCT TGG AAG CCC TAC-3', IL-5 forward 5'-TGA CAA GCA ATG AGA CGA TGA G-3', IL-5 reverse 5'-CCC ACG GAC AGT TTG ATT CTT C-3', IL-13 forward 5'-TCC AGC CTC CCC GAT ACC-3', IL-13 reverse 5'-AGC AAA GTC TGA TGT GAG AAA GG-3'.