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Mechanism of interleukin-25 (IL-17E)-induced pulmonary inflammation and airways hyper-reactivity

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Summary

Background IL-25, a novel member of the IL-17 cytokine family, promotes CD4⁺ T-helper 2 lymphocyte-like (Th type-2) inflammatory responses in the lung. Although IL-25 up-regulates IL-13 in the lung, the contribution of this and other type 2 cytokine signalling pathways to the induction and persistence of airways hyper-reactivity (AHR) and allergic inflammation are unclear.

Objective To determine the downstream factors employed by IL-25 to induce Th type-2 pulmonary inflammation and AHR.

Methods IL-25 was delivered to the airways of BALB/c mice by intra-tracheal (i.t.) instillation and AHR and Th type-2 inflammatory responses were characterized in wild type (WT) and Th type-2-cytokine and -signalling pathway-deficient (–/–) mice.

Results IL-25 treatment resulted in AHR, eosinophilic inflammation, mucus hypersecretion and a progressive increase in the production of Th type-2 cytokines in the lungs. Levels of arginase-I (arg-I) and eotaxin were also elevated by IL-25 treatment. A significant reduction in AHR, and attenuation of mucus production was observed in IL-25-treated IL-13^{–/–}, IL-4 receptor alpha (IL-4Rα^{–/–})- and signal-transducer-and-activator-of-transcription-factor-6 (STAT6^{–/–})-deficient mice. AHR was also inhibited in IL-4^{–/–} and IL-5/eotaxin(1)^{–/–} deficient mice treated with IL-25, however, mucus hypersecretion was not completely ablated. IL-25 promoted Th type-2 responses by directly acting on naïve T cells.

Conclusion IL-25 potently (single dose) induces sustained AHR and acute pulmonary inflammation with eosinophilia. IL-25-induced AHR is dependent on the production of Th type-2 cytokines, and removal of IL-13 and its signal transduction pathway prevents IL-25-induced airways inflammation and AHR. IL-25 potently induces inflammatory cascades that may exacerbate allergic airways inflammation by promoting Th type-2 cytokine responses in conjunction with the up-regulation of factors (eotaxin and arg-I) that can amplify inflammation associated with allergic disorders. Dysregulation in IL-25 production may predispose to features of allergic airways disease.

Keywords allergy/asthma, inflammation, interleukins, mouse model

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Introduction

IL-25 (IL-17E) is a member of the IL-17 family of cytokines. Six members of the IL-17 family have been identified based on structural homology to IL-17A [IL-17A-D, IL-17E (IL-25) and IL-17F]. The role of IL-17 family members, however, in the regulation of specific immune

responses and in the associated signalling mechanisms involved in the induction of inflammatory responses remain largely unknown [1, 2]. Members of the IL-17 family show specific and varying biological functions [3], which results from differences in the length and structure of N- and C-terminal regions, and in the number of cysteine residues. Diversities in receptor complexes,

downstream intracellular signalling pathways and expression profiles in various organs [1], have been predicted to account for the different biological activities of IL-17 family members. IL-17 cytokines may also be differentially regulated (spatially and temporally) by different immune and inflammatory responses.

IL-17A is known to up-regulate a range of pro-inflammatory molecules [tumour necrosis factor (TNF)- α , IL-1 β , IL-6, IL-8, monocyte chemo-attractant protein (MCP)-1 and granulocyte-macrophage colony stimulating factor (GM-CSF)], intracellular adhesion molecule (ICAM)-1 and promote CD4⁺ T-helper 1 lymphocyte (Th type-1) responses [4, 5]. IL-17B and IL-17C induce TNF- α and IL-1 β responses [6], IL-17D augments IL-6, IL-8 and GM-CSF activity [7], and IL-17C and IL-17F induce neutrophilia and the production of IL-6 and interferon γ (IFN- γ) [3]. By contrast, only limited investigations have linked IL-25 to Th type-2 immune responses and the regulation of allergic inflammation.

Human and mouse IL-25 share 80% identity [8]. Human IL-17E has two isoforms; isoform-1 has an extended N-terminal sequence and is expressed at high levels in the testis, trachea and brain [9] by comparison with isoform-2, which is mainly expressed in the testis, prostate and spleen [10]. IL-25 activates nuclear factor (NF)- κ B by binding to a newly identified IL-17Rh1 receptor, which is highly expressed in human kidney, liver and small intestine [9]. In mice, IL-25 is highly expressed in the gastrointestinal tract and uterus [8], and the IL-17Rh1 receptor is expressed in the lymphoid organs, lungs and liver [10]. *In vitro*, Th type-2 cytokines induce the expression of the IL-25 receptor in human macrophages, dendritic cells [11] and B cells [12] and the stimulation of murine T cells [8] and mast cells (activated by IgE) results in the secretion of IL-25 [13]. Instillation of IL-25 into the lungs of mice increases the production of Th type-2 cytokines (IL-4, IL-5, IL-13), total IgE and IgG1 antibody levels and the numbers of eosinophils in the bronchoalveolar lavage fluid (BALF), pulmonary tissue and blood [3, 8, 10, 14]. IL-25 is also upregulated during parasitic and fungal infections [3].

Collectively, investigations suggest that IL-25 may play a pivotal role in development of Th type-2 immune responses and thus may be a target for the suppression of allergic inflammatory cascades [3, 8]. Although previous investigations have demonstrated that IL-25 up-regulates IL-13 levels in the lung (a cytokine linked to the induction of AHR and allergic disease) the contribution of this pathway and other Th type-2 inflammatory cytokines to the induction and persistence of AHR and the allergic phenotype (eosinophilia and mucus hypersecretion) have not been determined. Our data show that IL-25 induces potent and sustained AHR and inflammation that is dependent on Th type-2 cytokines. AHR is associated with increased numbers of airway eosinophils and mucus

hypersecretion and the up-regulation of factors (eotaxin and arg-1) that can amplify inflammation associated with allergic disorders. IL-25 promoted Th type-2 responses by acting on naïve T-cells.

Materials and methods

Mice

BALB/c WT, IL-4R α ^{-/-} and IL-4^{-/-} [15], IL-13^{-/-} [16], STAT6^{-/-} [17] and IL-5/eotaxin^{-/-} [18]-deficient mice (6–9 weeks old) were bred in specific pathogen-free facilities at the Australian National University. Mice were either generated on a BALB/c background or crossed for at least 10 generations onto the BALB/c strain.

Intra-tracheal instillation of interleukin-25

Mice were anaesthetized with alfaxalone solution [10 mg/mL, Jurox, New Zealand, 100 μ L diluted 1 : 4 in sterile phosphate-buffered saline (PBS)] by tail vein injection. Murine recombinant IL-25 (rIL-25) [5 μ g (maximally effective dose), (DNAX), in 25 μ L of PBS] or PBS (as vehicle control) were instilled into the trachea via the larynx utilizing a 22-gauge needle catheter.

Measurement of airways hyper-reactivity

AHR was measured by barometric plethysmography as previously described [19]. Briefly, mice were placed into plethysmograph chambers and exposed to an aerosol for 2 min. Initially, mice were exposed to distilled water (diluent for methacholine) to obtain baseline readings. This was followed by exposure to aerosols of methacholine at doubling concentrations (3–50 mg/mL). The inlet was closed after each 2 min exposure for 3 min to record enhanced pause (Penh). Mean Penh values are reported as the percentage increase over baseline for the 25 mg/mL (maximally effective) dose but are reflective of the full dose-response curve. Although Penh is only a surrogate marker for the development of AHR, we have previously shown that changes in Penh in response to methacholine correlate with increased resistance in BALB/c mice [20].

Examination of bronchoalveolar lavage fluid and lung histopathology

BALF was collected in 1 mL of a 1 $\times$ PBS solution by infusion through the trachea. Cytospin slides were prepared and stained with May-Grünwald Giemsa and the types of leucocytes in BALF were differentiated by morphological criteria and enumerated. Lung tissue representing the bronchi, bronchiole and alveoli was fixed in 10% formalin, embedded in paraffin and sectioned. Deparaffinized sections were stained with Charbol's chromotrope

for detection of eosinophils and periodic acid–Schiff for identification of mucus. The number of eosinophils and mucus-secreting cells were identified by morphological criteria and enumerated per high-powered field (HPF) [21].

Detection of cytokines by enzyme linked immunosorbent assay

Briefly, 4×10^6 cells from the peribronchial lymph nodes (PBLN) were pooled from four mice or 4×10^7 lung cells were cultured in the presence or absence of rIL-25 (70 ng/mL) at 37 °C for 72 h. The culture supernatants were stored at –20 °C and later analysed for cytokines by ELISA as previously described [21, 22]. Limits of detection were 5 pg/mL for IL-4, 24 pg/mL for IL-5 and 12 pg/mL for IL-13.

Ovalbumin sensitization

Mice were intraperitoneally sensitized with 50 µg ovalbumin (Ova)/1 mg Alhydrogel (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia) in sterile saline as we have previously described [18, 21]. Control group was injected with saline and Alhydrogel. All groups of mice were challenged with Ova on days 12 and 14 by aerosol (aero-challenged) to induce allergic inflammation and peribronchial lymph nodes were (PBLN) isolated on day 16.

The direct effect of interleukin-25 on naïve or antigen-specific T cells

Two T cell preparations were used to determine the effects of IL-25 on T cell function (proliferation and cytokine secretion). The effect of IL-25 on the responsiveness of T cells in cell cultures derived from the PBLN of Ova-sensitized mice (antigen-specific cells) or saline-treated mice (naïve cells) to TCR engagement and co-stimulation with anti-CD3 (α CD3) and α CD28, respectively. PBLN were pooled from four naïve or sensitized mice and were cultured in MLC complete medium with α CD3 (50 ng/mL)/ α CD28 (1 µg/mL) in the presence or absence of IL-25 (70 ng/mL) for 72 h. T cell proliferation and cytokine production was then measured. Cytokines were analysed in culture supernatants by ELISA as described above. T cell proliferation was detected by using Cell Titer96 solution (as described by the manufacturer, Promega, Madison, WI, USA) and absorbance was recorded at 490 nm. Assays were performed in duplicate.

RNA isolation and reverse transcriptase-polymerase chain reaction

Pooled PBLN from four mice or individual lungs were suspended in 1 mL of RNazol (Geneworks PtyLtd, Hino-marsh, South Australia, Australia), quickly frozen on dry ice and stored at –70 °C. Total RNA was isolated accord-

ing to the manufacturer's instructions following aseptic procedures and 1 µg of total RNA was reverse transcribed [23]. PCR was performed in a 20 µL reaction mixture consisting of 10 × buffer, 10 ng cDNA, 5 mM dNTPs (Gibco Ltd, Mt Waverley, Victoria, Australia), 10 pm/µL of each of the specific primers, 25 mM MgCl₂ and 1 U Taq DNA polymerase (AB gene, Surrey, UK) at an annealing temperature of 55 °C. The primer sequences and PCR protocols utilized have been previously reported [23, 24]. For NF-κB primers for the p65 subunit, Rel A were employed: forward primer – tctgaaccagggtgtgtc; reverse primer – tgagagaccattgggaagc. The PCR products were visualized on 1% agarose gels containing ethidium bromide. Bands (arg-I and eotaxin) were normalized to the housekeeping gene, hypoxanthine–guanine phosphoribosyl transferase (HPRT) and analysed using Image analysis 4.02 software (Scion, Frederick, MD, USA).

Statistical analysis

Statistics were conducted using InStat 2.01 software (GraphPad, San Diego, CA, USA). Data represent mean values ± SEM. Parametric data were assessed by two-tailed unpaired Student's *t*-tests and non-parametric data were calculated by Wilcoxon or Mann–Whitney tests. *P* < 0.05 was considered as a significant difference between groups.

Results

Interleukin-25 induces allergic inflammation in wild type mice

Administration of IL-25 to the airways induced a potent and sustained AHR (Fig. 1a). AHR was induced within 24 h and was still detectable by day 16. The induction and maintenance of AHR correlated with the recruitment of eosinophils into the blood, airways and lung, and mucus hypersecretion in airway epithelial cells (Fig. 1b–f). Notably, AHR and tissue eosinophilia were greatest on day 16.

Interleukin-25 up-regulates T-helper type-2 cytokines, arg-I and eotaxin

The concentration of a range of factors that have been implicated in the development and persistence allergic inflammatory responses where measured after exposure to IL-25 (Fig. 2). Twenty four hours after exposure of the lung to IL-25, IL-5 and IL-13 in PBLN cultures (Fig. 2a–c), and IL-4, IL-5 and IL-13 in lung cell cultures (results not shown), were increased after *in vitro* stimulation with IL-25, when compared with similarly treated cells from mice only exposed to PBS. At 8 days, the levels of all Th type-2 cytokines examined were significantly

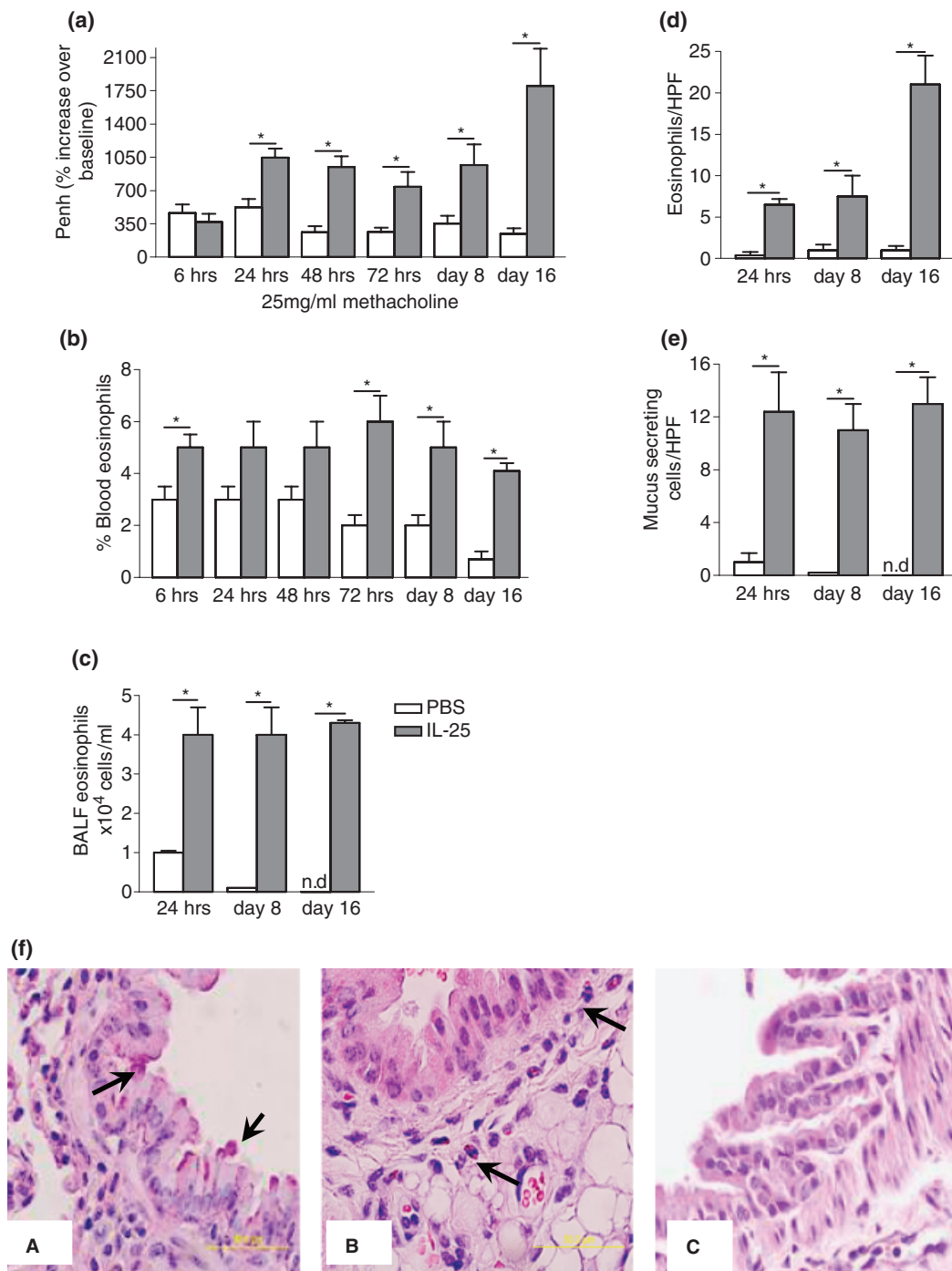


Fig. 1. IL-25-induced airways hyper-reactivity, eosinophilia and mucus hypersecretion in the lung. IL-25 or Phosphate buffer saline (PBS) was instilled into the trachea of wild type mice and assessment was performed over 16 days. (a) Airways hyper-reactivity responses at the maximal effective dose (25 mg/mL) of methacholine are only shown but are representative of responses obtained over the full dose response curve. Eosinophil numbers in (b) blood, (c) bronchoalveolar lavage fluid (BALF) and (d) tissue; (e) numbers of mucus secreting cells in the airways; (f) histopathology sections showing (A: arrows) mucus secreting epithelial cells and (panel B: arrows) tissue eosinophils after IL-25 treatment, and (c) control PBS treated tissue at 24 h. Results are mean $\pm$ SEM of four to six mice per group in two to three individual experiments. Significantly different (* $P < 0.05$) from control PBS-treated group.

increased in IL-25-stimulated cultures of PBLN and lung cells (results not shown) from IL-25-treated mice ($P < 0.05$). IL-5 and IL-13 levels were still significantly

increased on day 16 by comparison with the PBS group ($P < 0.05$). All Th type-2 cytokines were significantly increased in IL-25 stimulated PBLN and lung cell cultures

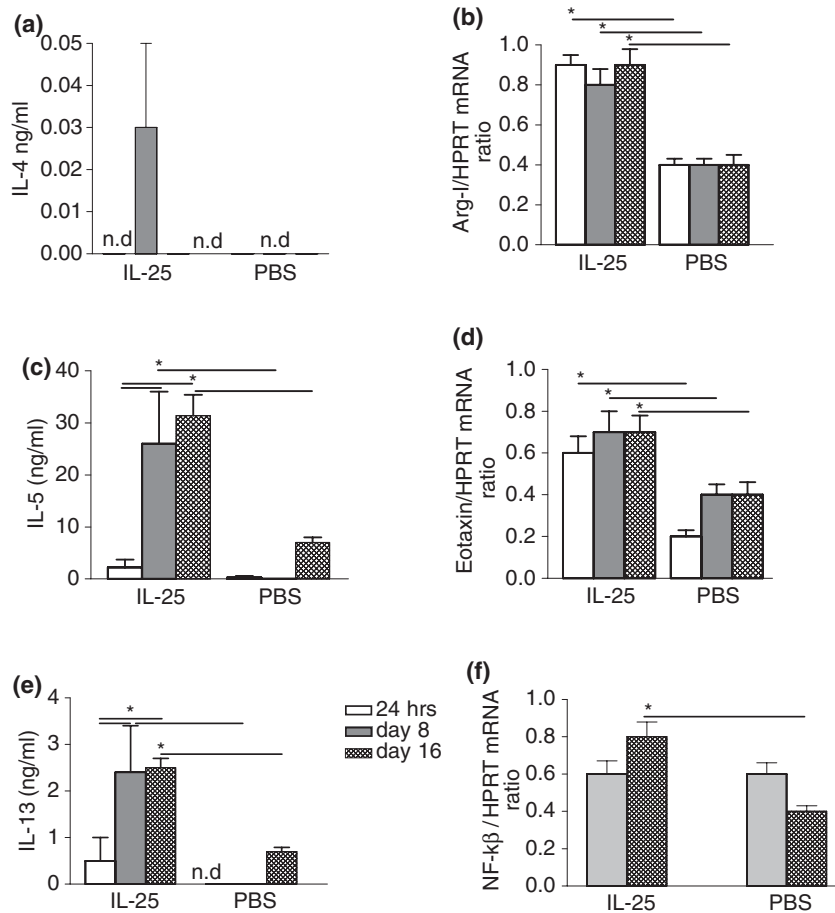


Fig. 2. IL-25 treatment induced the production of Th type-2 cytokines and enhanced the expression of eotaxin, arg-I and NF- κ B. Peribronchial lymph nodes or lung cells (results not shown) were isolated from mice 24 h and 8 and 16 days after intra-tracheal administration of IL-25 or phosphate buffered saline (PBS). Cells were then stimulated with IL-25 (70 ng/mL) in culture for 72 h. (a–c) Cytokine levels in the culture supernatants were determined by ELISA. A representative of duplicate experiments is shown. Levels of expression of arg-I, eotaxin, NF- κ B and control hypoxanthine-guanine phosphoribosyl transferase (HPRT) were determined by reverse transcription-polymerase chain reaction and results are shown as the (d) arg-I/HPRT, (e) eotaxin/HPRT and (f) NF- κ B/HPRT mRNA ratios (data for 8 and 16 days is only shown). Not detected (ND). Significantly different (* P < 0.05) from control PBS-treated group or IL-25 24 h group.

(results not shown) from IL-25-treated mice by comparison with similarly treated cells from PBS exposed mice (Fig. 2a–c).

Eotaxin, and arg-I were highly expressed in PBLN and lung cells (results not shown) stimulated with IL-25 *in vitro*, 24 h and 8 days after exposure to IL-25 but not in the PBS-treated group (Fig. 2d, e). NF- κ B was only increased 16 days after IL-25 exposure.

Mechanisms of interleukin-25 mediated airways hyper-reactivity and T-helper type-2 inflammation

Pulmonary exposure of WT mice to IL-25-induced AHR in response to methacholine (Fig. 3a). However, in the absence of Th type-2 cytokines or molecules involved in their signal transduction pathways, IL-25 did not induce pronounced AHR compared with PBS-treated mice. Eosinophil migration from the lung to the airway lumen

(BALF) in response to IL-25 was significantly decreased in all factor-deficient mice (P < 0.05, Fig. 3b). A reduced, but still significant tissue eosinophilia was observed in IL-13 $^{-/-}$, STAT6 $^{-/-}$ and IL-4R α $^{-/-}$ mice (Fig. 3c). By contrast, eosinophils were absent in IL-5/eotaxin $^{-/-}$ mice but not significantly reduced in IL-4 $^{-/-}$ mice after IL-25 treatment. Thus, migration of eosinophils from lung tissue into the airway lumen in response to IL-25 exposure is critically dependent on IL-4 (Fig. 3b, c). Furthermore, IL-25-induced AHR is not dependent on eosinophil accumulation in the lung, as AHR was abolished in IL-4 $^{-/-}$ mice. Mucus production was not induced in IL-13 $^{-/-}$, STAT6 $^{-/-}$ and IL-4R α $^{-/-}$ mice (Fig. 3d) in response to IL-25 treatment. In addition, mucus production was still observed in the absence of eosinophils (IL-5/eotaxin $^{-/-}$ mice) and IL-4 $^{-/-}$. Thus, IL-25-induced mucus production is critically linked to IL-13 and signalling through the IL-4R α chain and STAT6.

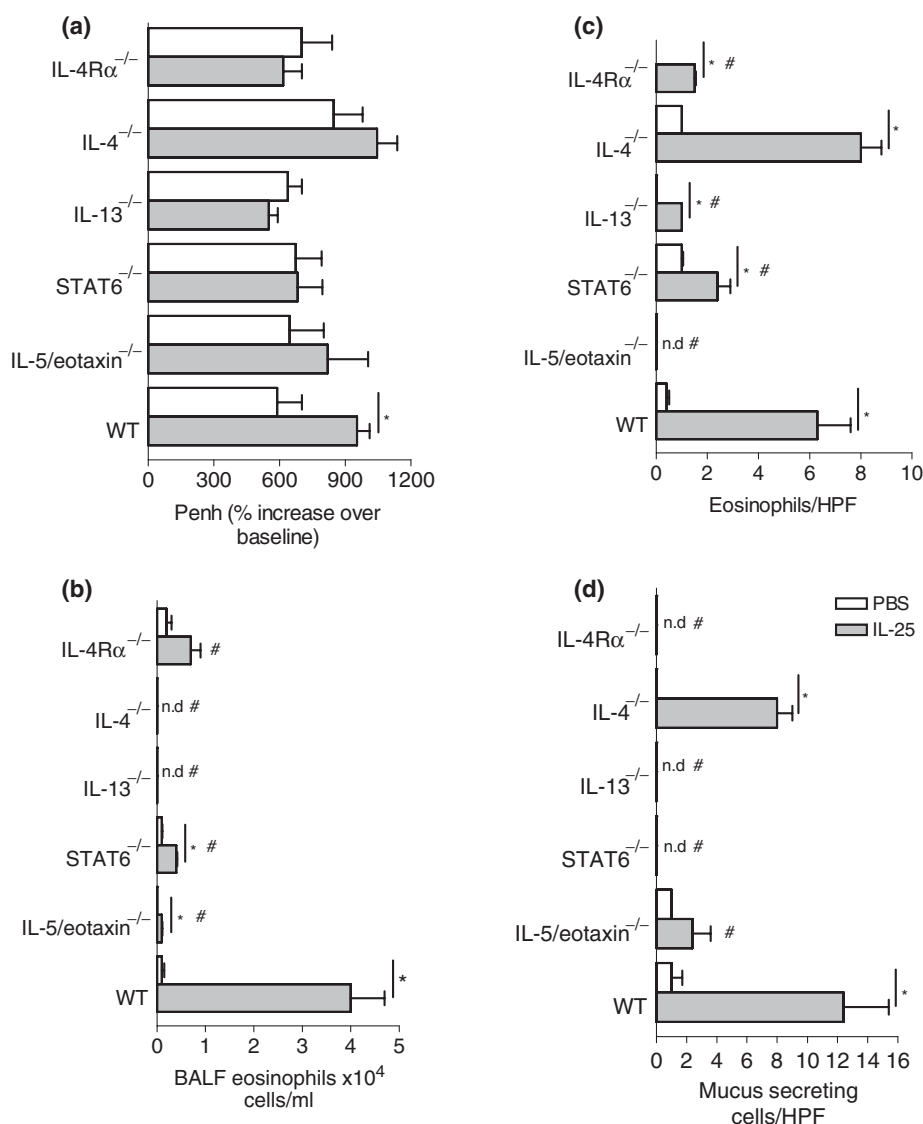


Fig. 3. The effect of IL-25 on the induction of airways hyper-reactivity, eosinophilia and mucus hypersecretion in mice deficient in Th type-2 cytokines and signalling pathways. IL-25 or phosphate buffer saline was instilled into the trachea of mice and assessment was performed 24 h later. (a) Airways hyper-reactivity responses at the maximal effective dose (25 mg/mL) methacholine are only shown but are representative of responses obtained over the full dose-response curve. Numbers of eosinophils in the (b) Bronchoalveolar lavage fluid (BALF) and (c) tissue, and (d) numbers of mucus-secreting cells in the airways are shown. Data are reported as mean $\pm$ SEM of four to six mice per group and are representative of two to three individual experiments. Not detected (ND). Significantly different (* P < 0.05) from respective control PBS-treated group or from IL-25-treated wild type mice (# P < 0.05).

The direct effect of interleukin-25 on naïve or antigen-specific T cells

The effect of IL-25 on the responsiveness (proliferation and cytokine secretion) of antigen specific or naïve T cells in PBLN cell cultures to stimulation with α CD3 and α CD28 was determined (Fig 4). Treatment of naïve but not antigen-specific memory T cells with IL-25 enhanced their ability to proliferate in response to stimulation (Fig. 4a). Treatment of naïve T cells with IL-25 also increased their capacity to secrete IL-4, IL-5 and IL-13 but not IFN- γ (Fig. 4b–e). IL-25 only significantly promoted the produc-

tion of IL-4 in antigen-specific T cells although a trend to increased production of IL-5 and IL-13 was observed.

Discussion

In this investigation we show that IL-25 potently (one dose to the airways) induces AHR and inflammatory cascades that are driven by Th type-2 cytokines, which are associated with allergic airways inflammation. Notably, the AHR and inflammatory cascades (characterized by increased numbers of eosinophils in the airways and lung

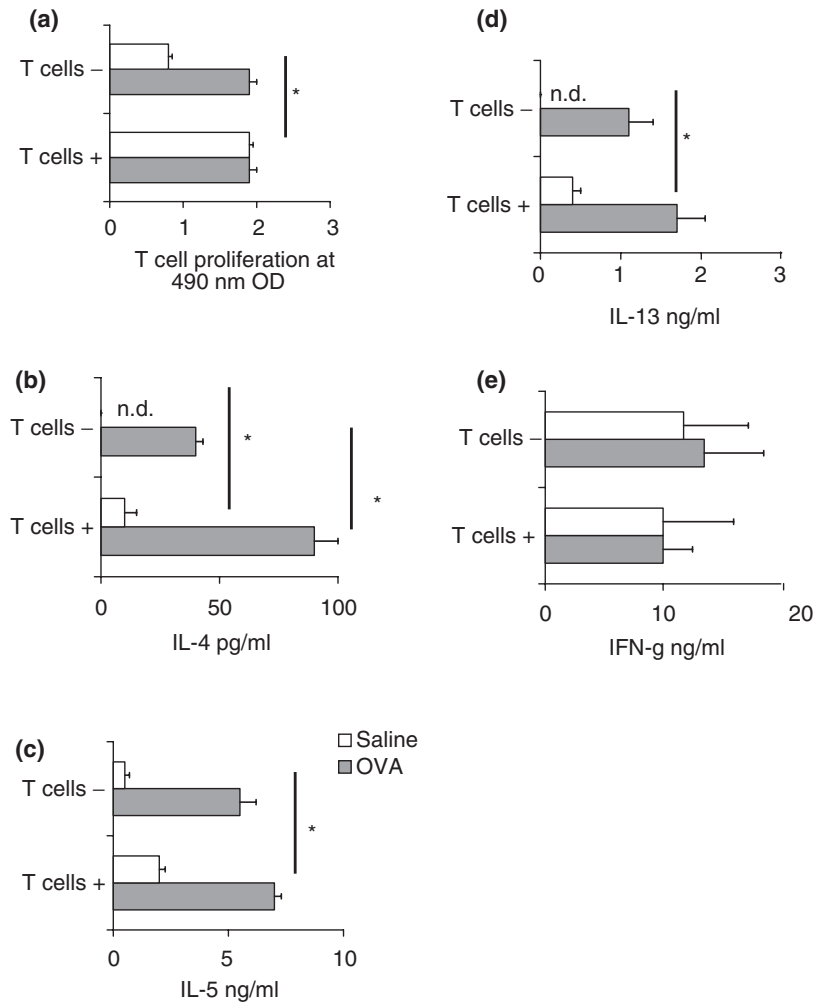


Fig. 4. IL-25 induces increased proliferation and Th type-2 cytokine secretion of naïve peribronchial lymph nodes T cells. The effect of IL-25 on the responsiveness (proliferation and cytokine secretion) of naïve (saline group, clear bars) or antigen-specific T cells (OVA group, shaded bars) in PBLN cell cultures were stimulated with α CD3 and α CD28 in the presence [denoted as positive (+)] or absence [denoted as negative (–)] of IL-25 and proliferation rate (a) and cytokine secretion (b–e) characterized. PBLN were pooled from four naïve or sensitized mice and were cultured in MLC complete medium with α CD3 (50 ng/mL)/ α CD28 (1 μ g/mL) in the presence or absence of IL-25 (70 ng/mL) for 72 h. T cell proliferation and cytokine production in culture supernatants were then measured using CellTiter96 solution or ELISA, respectively, as described in Materials and methods. Assays were performed in duplicate significantly different (* P < 0.05) from groups indicated.

tissue, and mucus hypersecretion) developed rapidly and were sustained for at least 16 days.

The potent and sustained effects of IL-25 were similar to those we have previously observed by delivery of recombinant IL-13 to the lung [20] suggesting that IL-13 played a key role in IL-25-mediated responses. Indeed, like IL-13 [20], IL-25 rapidly promoted (within 24 h) eosinophil recruitment to the lung, mucus hypersecretion, and eotaxin and arg-I expression that was sustained for 16 days. Exposure of PBLN or lung cells to IL-25 in culture resulted in the production of IL-13 [20]. Here, we also show that IL-25-dependent induction of AHR, eosinophilia (in part) and mucus hypersecretion are mediated by IL-13 and its downstream signalling pathways (STAT6 and IL-4R α). Thus, IL-13 plays a key role in mediating the

downstream effects of IL-25. By contrast to the effect of IL-25, IL-13 induces AHR that persists for only 96 h [20] further highlighting the potency of IL-25 in modulating immune responses that predispose to persistent AHR. The factors promoting persistent AHR in asthma remain unknown. IL-13 levels were still elevated 16 days after IL-25 administration. It will be of interest to determine whether IL-25 promotes sustained AHR and inflammation by maintaining the levels of IL-13 and other type-2 cytokine levels and/or by inhibiting factors that promote resolution of IL-13-mediated effects.

IL-25 up-regulated the expression of eotaxin and arginase 1. Eotaxin, with IL-5, plays a key role in the recruitment of eosinophils into the airways in asthma [18, 25]. Recently, we made the novel observation that

arginase-1 activity is up-regulated in asthma and in a number of experimental models of this disorder [24]. L-arginine is catabolized by inducible nitric oxide (NO) synthase (iNOS) to promote the production of NO, while a range of second messengers are generated through arginase-1 activation. iNOS and arginase-1 activity are differentially modulated by Th type-1 and Th type-2 cells and cytokines, respectively [26, 27]. In particular, IL-4 and IL-13 inhibit NO production in macrophages through substrate depletion by activating arginase-1 in a STAT6-dependent manner [27]. Diversion of L-arginine metabolism away from NO production may have important implications for the regulation of airways responsiveness and in the development of chronic diseases. NO is bronchodilatory and L-arginine is catabolized to proline and polyamines that regulate collagen production and cell proliferation [28]. The up-regulation of arginase 1 and IL-13 activity in PBLN and resident lung cells suggests a potential role for IL-25 in the upstream regulation of AHR and potentially airway wall remodelling and obstruction associated with asthma.

AHR was attenuated in the absence of IL-4 and IL-5/eotaxin, where tissue eosinophilia persists and is absent, respectively, after exposure to IL-25. These data suggest that eosinophils *per se* are not directly involved in IL-25-mediated AHR. Notably, the magnitude of the eosinophil influx is much smaller than that observed in models of antigen-induced asthma, which reflects a response to a single challenge of cytokine rather than the complex signals elicited by T cell activation. We have previously shown that eosinophils regulate AHR, but this only pertains to models of allergic airways disease where this cell regulates T cell production of IL-13. In models of cytokine induced AHR, such as with recombinant IL-13 or IL-25, tissue eosinophils can often be dissociated from the persistence of AHR. This indicates that pathways downstream of the eosinophil or those not requiring contributions from eosinophils are activated directly by the recombinant cytokines. Notably, in the absence of IL-4 tissue eosinophilia was not decreased while their numbers in the airway lumen were significantly reduced. This supports previous data in allergic models of asthma that this cytokine plays a pivotal role in regulating the movement of eosinophils across the airway epithelium [29]. We speculate that this process may be related to the ability of IL-4 to up-regulate chemotactic factors for eosinophils (such as eotaxin) in the airway wall.

Exposure of PBLNs and lung cells from IL-25-treated mice to IL-25 in culture demonstrated the capacity of this cytokine to prime leucocyte and resident pulmonary cells for enhanced production of Th type-2 cytokines. Previous studies have also shown that IL-25 can promote increases in total serum IgE and activate NF- κ B in cells, which is linked to transcription regulation of cytokines [3]. Thus, dysregulation of IL-25 production that results in elevated

levels in the lung may induce hallmark features of allergic airways disease independently of T cell activation. However, in this investigation we show that IL-25 can promote Th2 responses directly by amplifying proliferation and type-2 cytokine production from naïve T cells in PBLN cultures. Notably this effect was more pronounced on naïve T cells rather than antigen-specific T cells. This data suggests that stimulation of the production of IL-25 early after antigen exposure will help drive the adaptive T cell response to a Th type-2 phenotype.

In summary, IL-25 may play a key role in the development and exacerbation of asthma and allergic inflammation by promoting Th type-2-like responses in the airways, in part by acting directly on naïve T cells to promote the Th2 phenotype. Notably IL-25 induces sustained AHR and inflammatory cascades by acting upstream of Th type-2 cytokine gene regulation. Thus, IL-25 is potentially an important therapeutic target in suppressing Th type-2 immune responses in asthma and allergy.

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