

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Myeloid dendritic cells are primed in allergic asthma for thymic stromal lymphopoietin-mediated induction of Th2 and Th9 responses

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Keywords

asthma; interleukin-9; myeloid dendritic cells; Th9; thymic stromal lymphopoietin.

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Abstract

Background: Type 1 myeloid dendritic cells (mDCs) contribute to inception of allergic asthma (AA) and are regulated by epithelial-derived cytokines.

Objectives: To evaluate whether mDCs from AA patients are primed for thymic stromal lymphopoietin (TSLP)-driven responses.

Methods: mDCs from 18 AA patients and 15 controls were purified using immunomagnetic sorting. Cells were pulsed with TSLP or *Dermatophagoides pteronyssinus* (Der p) allergen, before FACS phenotyping and co-culture with allogeneic CD4⁺ T cells. Bronchial biopsies from 15 AA patients and four controls were immunostained for CD1c and TSLP receptor (TSLPR).

Results: Allergic asthma patients had a higher proportion of TSLPR⁺ mDCs, in blood and bronchial mucosa. When compared to mDCs from controls, both TSLP- and Der p-pulsed blood mDCs from AA patients induced increased polarization of CD4⁺ T cells into Th2 cells (IL-5, IL-13, and GATA3⁺), while only TSLP-mDCs promoted Th9 cells (IL-9 and PU.1⁺/IRF4⁺). In addition, OX40L was induced upon TSLP stimulation and was required for the induction of Th2, but not Th9, cells. In contrast, development of Th9 cells in this model depended on TGF-β1.

Conclusions: Our data indicate overlapping but partially distinct effects of TSLP and Der p allergen pathways, showing that DCs are primed in human asthma for TSLP-driven induction of both Th2 and Th9 cells. This novel TSLP/mDC/Th9 axis operates through a distinct, OX40L-independent pathway. These data further highlight the TSLP pathway as a relevant target in human asthma.

Besides their ability to sample antigens, myeloid dendritic cells (mDCs) are able to migrate to regional lymph nodes, stimulate and polarize effector T-cell responses (1, 2). mDCs can be divided into two subtypes: type 1 mDCs, which

express BDCA1 [0.60% of peripheral blood mononuclear cells (PBMC)], and type 2 mDCs, a small population (0.03% of PBMC) characterized by the expression of BDCA3 and a lower capacity to stimulate T-cell proliferation (3).

In human asthma, it has been shown that peripheral blood DCs are rapidly recruited from blood to the airways (4, 5). Their function is locally modulated by epithelial cytokines, including interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP) (6–9).

Thymic stromal lymphopoietin is an IL-7-related cytokine involved in T-cell maturation and Th2 induction (10). Thymic stromal lymphopoietin ligation to its heterodimeric

Abbreviations

AA, allergic asthma; BALF, bronchoalveolar lavage fluid; Der p, *Dermatophagoides pteronyssinus*; FeNO, exhaled nitric oxide fraction; FEV1, forced expired volume in 1 s; LPS, lipopolysaccharide; mDC, myeloid dendritic cell; MLR, mixed leukocyte reaction; OX40L, OX40 ligand; TSLP, thymic stromal lymphopoietin; TSLPR, receptor to thymic stromal lymphopoietin.

receptor on mDCs enhances the expression of the costimulatory molecule OX40L (10, 11), which binds to OX40 on CD4⁺ T cells to induce Th2 differentiation.

While pulsing type 1 mDCs from allergic asthma patients with Der p allergen promotes Th2 responses (12–14), one group showed that TSLP could also promote the differentiation into Th9 cells, through a direct action on T cells expressing TSLPR (15). Th9 cells are a recently described T-cell subset playing pleiotropic activities in allergic inflammation (reviewed in 16, 17), and IL-9 might be a promising target for future therapies in asthma (18). It remains, however, unclear to which extent pro-allergic activities of inhaled allergens and epithelial-derived TSLP overlap at the level of DCs, for their regulation of T-cell responses.

In this study, we aimed to investigate whether type 1 mDCs from allergic asthmatics are primed to respond to TSLP for the induction and regulation of allergic T-cell responses. We assessed TSLPR expression on blood and bronchial mDCs from allergic asthma (AA) patients or controls and evaluated the reactivity of type 1 mDCs to TSLP in terms of polarization of CD4⁺ T cells, comparing findings to those obtained with Der p allergen and lipopolysaccharide (LPS).

Material and methods

For details, see online supporting information (Data S1).

Patients and controls

We enrolled 18 patients with mild to moderate AA, according to GINA (19), and 15 nonallergic, nonasthmatic controls (Table S1).

Bronchial biopsies were obtained from 15 asthma patients (nine with severe asthma and six with mild to moderate asthma) and four nonsmoking, nonallergic controls (Table S2). Endobronchial biopsies were collected as previously described (20), and tissue sections were immunostained for CD11c (Fast red, red) and TSLPR (DAB, brown).

The study was performed after obtaining approval from the local ethical committee and written informed consent from each subject.

Cell purification

Subjects underwent leukapheresis to obtain a buffy coat, and PBMC were obtained after centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). Type 1 mDCs were isolated using CD11c Dendritic Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). For co-culture experiments, CD4⁺ T cells from nonasthmatic donors were isolated using the CD4⁺ T cells Selection Kit (Miltenyi Biotec).

Statistical analysis

FACS data were expressed as percentage of positive cells, compared to isotype control. For nonparametric data, multiple comparisons were made with Kruskal–Wallis test

followed by Dunn's multiple comparison test. For single comparisons, unpaired data were analyzed by Mann–Whitney *U*-test and paired data by Wilcoxon matched pairs test. A *P* value under 0.05 was considered as statistically significant. Analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Statistics were reviewed by the *Support en Méthodologie et Calcul Statistique*, Université catholique de Louvain, Louvain-la-Neuve, Belgium.

Results

Allergic asthma patients have a higher proportion of TSLPR-expressing mDCs

Type 1 myeloid DCs were identified in FACS by the co-expression of CD11c, CD11b, and HLA-DR. The expression of costimulatory molecules CD80 and CD86 was also assessed (Fig. S1).

Allergic asthma patients had a higher proportion of blood TSLPR⁺ mDCs, as compared to controls ($28.85 \pm 16.65\%$ vs $5.76 \pm 5.27\%$, $P = 0.0012$; Fig. 1A).

The ratio of CD11c⁺ DCs expressing TSLPR was evaluated in bronchial tissue (Fig. 1B). Although density (number per mm²) of mDCs was similar in asthmatics and controls, proportion of TSLPR⁺ mDCs was significantly higher in asthma ($65.24 \pm 8.50\%$ and $67.51 \pm 14.17\%$ in mild and severe asthmatics, respectively) as compared to controls ($31.55 \pm 9.43\%$, $P = 0.0028$, Fig. 1C).

TSLP-educated mDCs from AA patients drive CD4⁺ T cells for Th2 and Th9 polarization

We addressed the reactivity of mDCs to TSLP, using T-cell regulation as readout. First, mDCs from AA patients or controls were pulsed for 48 h with TSLP (50 ng/ml), Der p extract (50 µg/ml), LPS (1 µg/ml), or a combination. DCs were then washed and cultured for 5 days with allogeneic CD4⁺ T cells from nonasthmatic, nonallergic donor in a paired manner [mixed leukocyte reaction (MLR) with the same T cells for one allergic asthmatic and one control].

To evaluate the effects of co-culture on T-cell phenotype, we studied the expression of CD45RA, CD45RO, and CD25 in CD4⁺ T cells at days 1, 3, and 4 of the MLR (Fig. S2). Besides confirming high purity of T cells (Fig. S2A), we showed that in the presence of mDCs, the proportion of CD45RA⁺ naïve T cells fell from 54% to 31% at the end of the MLR, while number of activated CD25⁺ CD4⁺ T cells rose from 6.2% to 51.5% (Fig. S2B and C).

Following pre-incubation with TSLP, mDCs from asthmatics significantly induced IL-5 (from 0.54 ± 0.36 to 2.03 ± 0.95 ng/ml, $P = 0.0159$) and IL-13 responses (from 0.34 ± 0.07 to 0.98 ± 0.43 ng/ml, $P = 0.0079$; Fig. 2). No change in IFN-γ or IL-10 was observed. This cytokine profile was very similar to that observed following mDC pulsing with Der p allergen (Fig. 2A). However, only TSLP-mDCs triggered IL-9 production (from 0.25 ± 0.11 to 0.76 ± 0.21 ng/ml, $P = 0.0079$; Fig. 2A). Combining TSLP

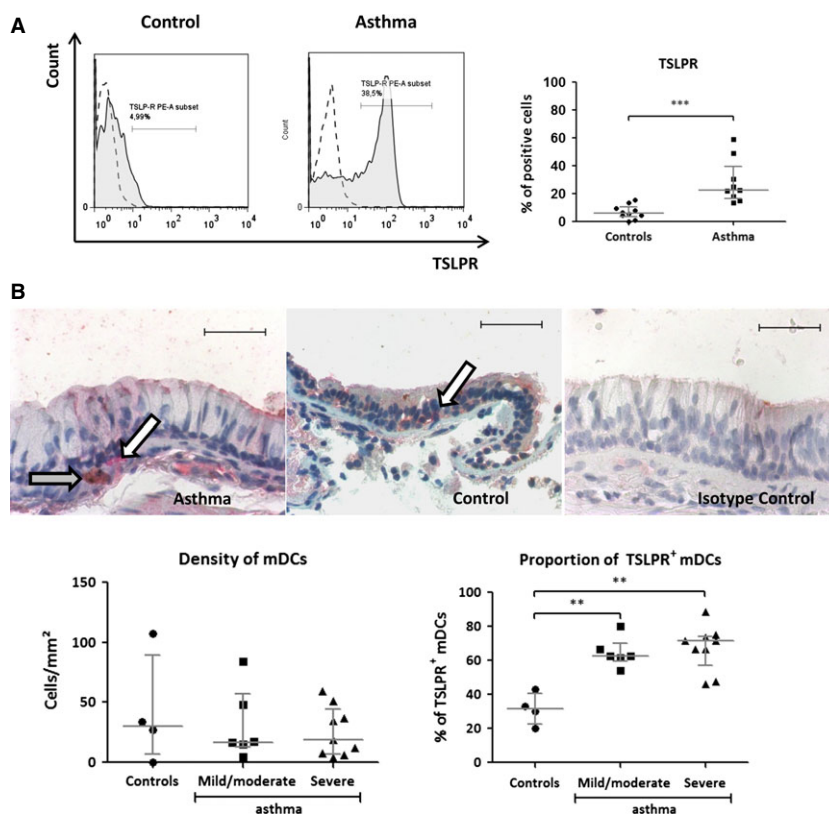


Figure 1 Upregulation of TSLPR in mDCs from patients with allergic asthma (AA). (A) AA patients ($n = 9$) have a higher proportion of peripheral blood TSLPR⁺ mDCs compared to controls ($n = 10$); representative FACS histograms are shown. Data are mean and interquartile range; *** $P < 0.001$ vs controls. (B) Immunohistochemistry on bronchial biopsies for CD1c (red) and TSLPR (brown). The white arrows show CD1c⁺ TSLPR⁻ mDCs, and the gray arrow shows a

CD1c⁺TSLPR⁺ mDC. Examples of one asthma and one control patient are shown, as well as the isotype control; bars represent 50 μ m. The density of bronchial mDCs is similar between the three groups (left graph), but the proportion of TSLPR⁺ mDCs is significantly increased in mild to moderate and severe asthma as compared to controls (right graph); data are mean and interquartile range, ** $P < 0.01$ vs controls.

and Der p did not increase cytokine responses, suggesting either overlapping pathways or maximal effects obtained with these concentrations. We then repeated MLR using mDCs from AA patients stimulated with either Der p, TSLP, or both in a dose-dependent manner (Fig. 2B). We showed that IL-5 and IL-13 responses increased with the dose of TSLP (up to 50 ng/ml) or Der p (up to 50 μ g/ml). Except for IL-13 at intermediate concentrations of TSLP and Der p, no clear additive or synergistic effect could be observed. In addition, these experiments confirmed that TSLP-mDCs were able to induce IL-9 secretion even at low concentrations of TSLP (Fig. 2B, right graph).

To confirm the induction of Th2 cells and Th9 cells by TSLP-mDCs from asthmatics, we studied the expression of the transcription factors T-bet, GATA3, IRF4, and PU.1 (Fig. 3), respectively, involved in Th1, Th2, and Th9 lineage differentiation. GATA3 was induced after 72 h in CD4⁺ T cells co-cultured with mDCs from asthmatics primed with TSLP or Der p (17.4% and 12.3% of positive cells, respectively). A subpopulation of CD4⁺ T cells primed with TSLP-mDCs expressed the transcription factors IRF4 and PU.1,

implicated in Th9 differentiation (10.9% and 14.2% of positive cells, respectively). As control for Th1 responses, LPS-mDCs could induce the expression of T-bet in CD4⁺ T cells (4.8% of positive cells).

Altogether, these data indicated that TSLP could imprint mDCs for T-cell regulatory properties which overlap those of Der p allergen (for Th2 responses), but also differ (for Th9 response).

mDCs from AA patients upregulate TSLPR and OX40L upon TSLP stimulation

In order to explore the underlying mechanisms linking TSLPR upregulation in mDCs from allergic asthmatics to Th2 induction, OX40L expression was assessed after 48 h of TSLP stimulation. TSLP stimulation of mDCs significantly increased the expression of OX40L in both groups (Fig. 4), with a significantly larger effect in asthmatics' mDCs ($P = 0.0159$). In addition, TSLP stimulation enhanced the expression of TSLPR itself, as a positive feedback loop, in both groups ($P = 0.0079$; Fig. 4).

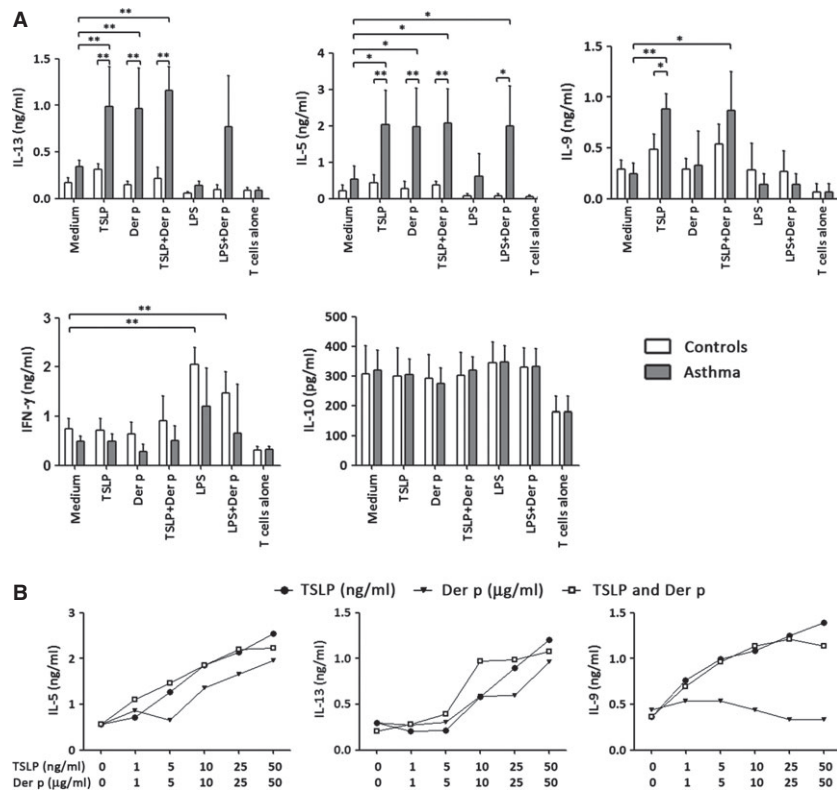


Figure 2 Thymic stromal lymphopoietin (TSLP)-pulsed mDCs from asthma patients drive Th2 and Th9 responses. (A) Secreted cytokines at the end of the allo-MLR with CD4⁺ T cells, according to the stimulation applied to mDCs. TSLP-mDCs induce the secretion of IL-5, IL-13, and IL-9; Der p-mDCs induce the secretion of IL-5 and IL-13; lipopolysaccharide (LPS)-mDCs induce IFN- γ secretion (and reduced IL-13, symbol not shown on the graph). IL-10 is unaffected. Data from five controls and five asthma patients

which were paired for the T-cell donor are mean $\pm$ SD. * P < 0.05, ** P < 0.01 vs medium (unstimulated mDCs) or vs control group, as indicated. (B) Priming of mDCs from asthma patients with Der p, TSLP, or a combination induces the secretion of IL-5, IL-13, and IL-9 in CD4⁺ T cells in a dose-dependent manner. There is induction of IL-5 and IL-13 from 10 ng/ml TSLP or 10 μ g/ml Der p. IL-9 induction by mDCs is already observed at 5 ng/ml of TSLP.

Blocking OX40L abolishes IL-5 and IL-13 responses, but not IL-9 response

Using mDCs from AA patients, the addition of a blocking mAb to OX40L (10 μ g/ml) during pulsing and co-culture abolished the induction of IL-5 and IL-13 by TSLP-mDCs, but not by Der p-mDCs (Fig. 5). However, anti-OX40L failed to inhibit IL-9 induction by TSLP-mDCs (Fig. 5). In the presence of neutralizing Ab to IL-2 (10 μ g/ml), production of IL-5, IL-13, and IL-9 was almost completely. When DCs were cultured in the presence of anti-TGF- β 1 (10 μ g/ml), IL-9 was inhibited, whereas IL-5 and IL-13 were not affected (1.85 and 1.06 ng/ml, respectively), as compared to control IgG (Fig. 5).

Discussion

Our study shows that type 1 mDCs from patients with AA display an increased expression of TSLPR in blood and in bronchial tissue. We showed that whereas Der p-mDCs promoted Th2 activation, TSLP-mDCs from asthmatics were able to

drive *in vitro* the development of both Th2 and Th9 cells and that this regulation operates through distinct pathways.

Thymic stromal lymphopoietin is an IL-7-related cytokine driving Th2 immune responses through the induction of expression of OX40L on DCs (10, 21). TSLP gene variant has been associated with AA and airway hyper-responsiveness (22, 23). Thymic stromal lymphopoietin expression is induced in the airways from patients with severe asthma (24), correlating with eosinophilia and Th2 inflammation. Interestingly, TSLP expression precedes the recruitment of DCs expressing TSLPR in the skin of allergic subjects upon intra-dermal challenge (25). We found a significantly higher proportion of TSLPR-expressing mDCs in the blood of allergic asthmatics compared to controls, a finding that was also observed locally within the bronchial mucosa of patients with mild or severe asthma. In addition, we show that TSLP-primed mDCs obtained from these patients are primed, upon TSLP pulsing, to induce IL-5, IL-13, and IL-9 responses in allogeneic CD4⁺ T cells. Interestingly, although Der p-educated mDCs from these patients could also induce Th2 cytokines, they could not induce IL-9.

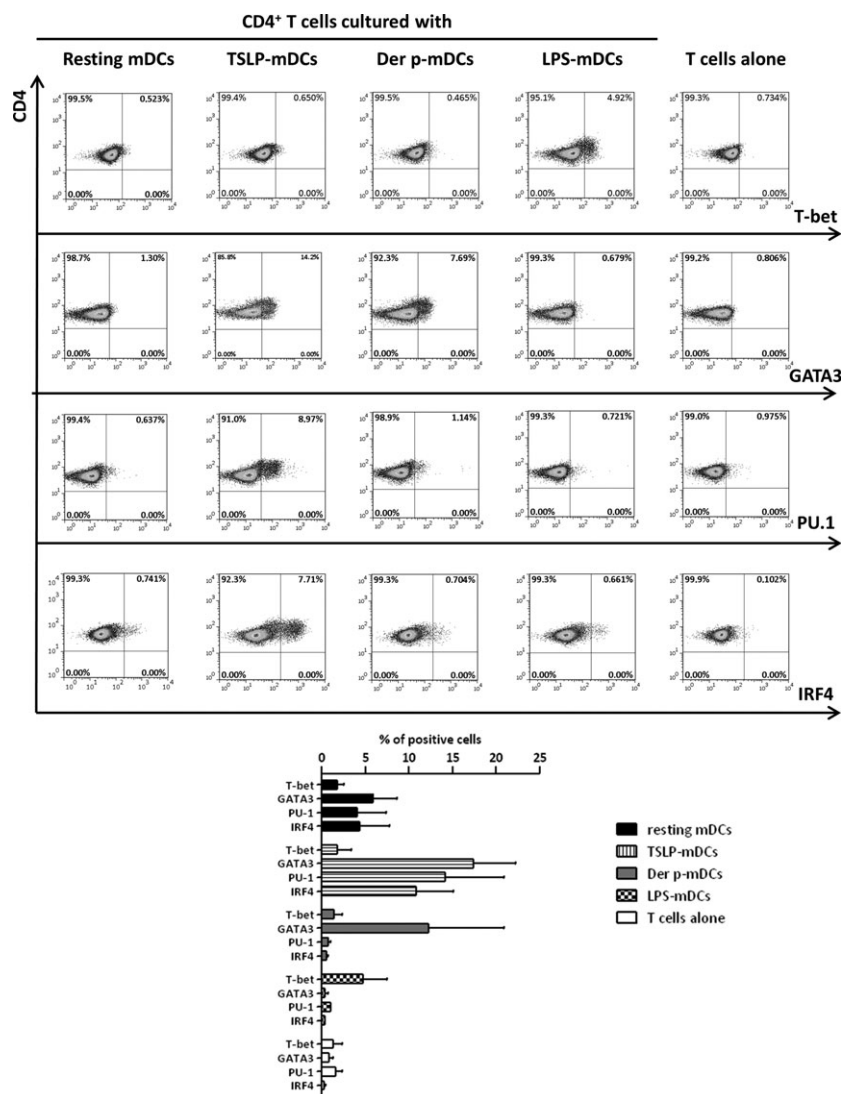


Figure 3 Expression of T-bet, GATA3, PU.1, and IRF4 transcription factors in CD4⁺ T cells cultured with peripheral blood mDCs from allergic asthma (AA) patients, according to the pulsing condition applied to mDCs for 2 days. Thymic stromal lymphopoietin

(TSLP)-mDCs enhance GATA3, PU.1, and IRF4 expression in CD4⁺ T cells; Der p-mDCs enhance GATA3; lipopolysaccharide (LPS)-mDCs enhance t-Bet. The lower graph shows data of three independent experiments.

The link between TSLP and IL-9 induction was first reported by Yao and colleagues (15) who showed that TSLP could promote Th9 differentiation *in vitro*, through a direct action on TSLPR⁺ CD4⁺ T lymphocytes. In addition, IL-9 increased in turn the expression of TSLPR on T cells (15). Here, we show that TSLP can also generate Th9 cells through its action on mDCs, as evidenced by the induction of T cells with Th9-related cytokine and transcription factor signatures (IL-9, IRF4/PU.1). Interestingly, it was shown that suppression of Th2 responses upon TSLP blockade *in vivo* was mediated through the modulation of mDCs (26). In addition, a pivotal role of OX40 signaling was reported for the development of Th9 cells in mice (27), showing that OX40 expression favored Th9 differentiation *in vivo*. In contrast, our data

indicate that the TSLP/mDC/Th9 axis does not depend on OX40L expression by DCs, as a blocking Ab to OX40L failed to suppress IL-9 response in our *in vitro* model. This apparent discrepancy between our results and those obtained by Xiao and colleagues could relate to differences in species (human vs mice) and/or in the experimental design (blocking Ab vs over-expressing OX40L in DCs). Unlike Th9, the induction of Th2 responses through TSLP-mDCs did depend on OX40L, as previously reported *in vitro* (10) and *in vivo* (21), suggesting that TSLP operates through distinct pathways in human mDCs to activate Th2 and Th9 responses. Our data show that induction of Th2 responses by TSLP or Der p allergen occurs through OX40L-dependent or independent pathways, respectively. The proposed pathways underlying Th2 and Th9

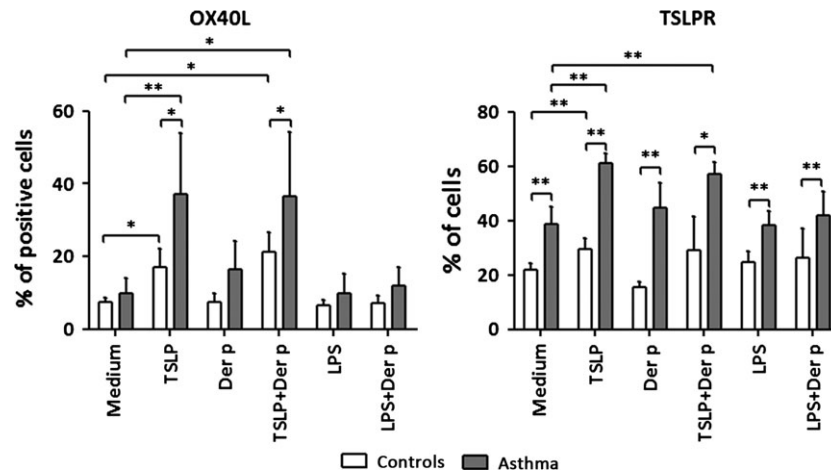


Figure 4 Upregulation of OX40L and TSLPR upon thymic stromal lymphopoietin (TSLP) stimulation of mDCs. Stimulation with high-dose TSLP (50 ng/ml) enhances the expression of OX40L and TSLPR on mDCs. Data from five controls and five asthma patients are

mean $\pm$ SD; * P < 0.05, ** P < 0.01 vs medium (unstimulated DCs) or vs control group, as indicated. Baseline levels of TSLPR are increased in asthma, as described in Fig. 1.

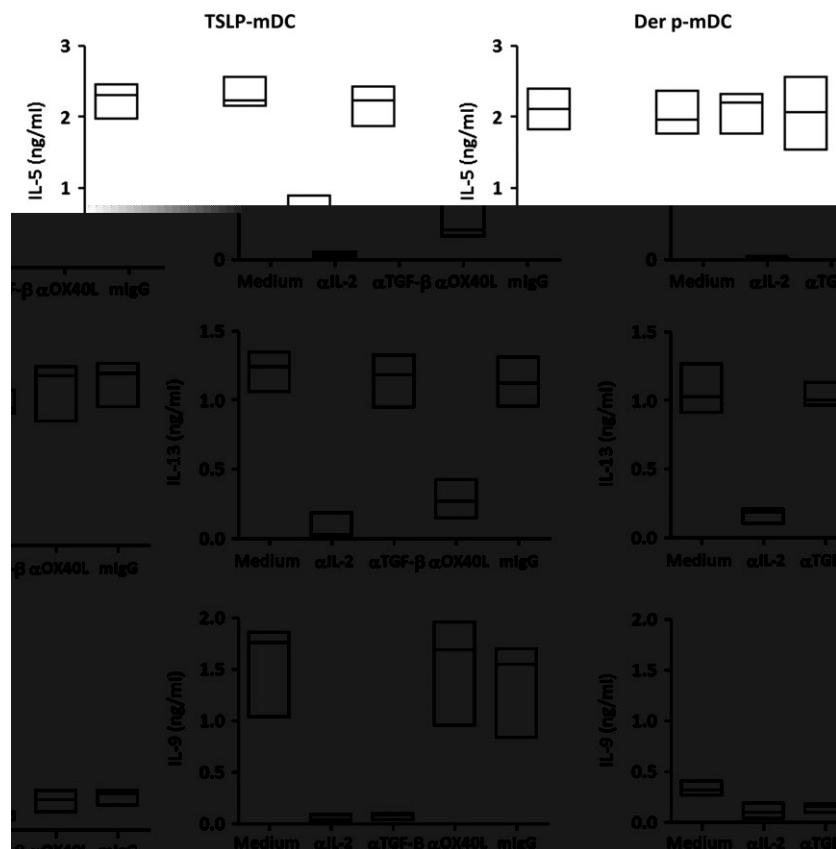


Figure 5 Role of OX40L for Th2 and Th9 polarization driven by thymic stromal lymphopoietin (TSLP)-mDCs. Although anti-OX40L antibody inhibited TSLP-mDC-driven IL-5 and IL-13 responses, it failed to abolish IL-9 induction. Anti-IL-2 blocked IL-5, IL-13, and IL-9

induction, whereas anti-TGF-β1 blocked IL-9, but not IL-5 and IL-13, upregulation. Data are whiskers plots (median and min. to max.) of three independent experiments with triplicates.

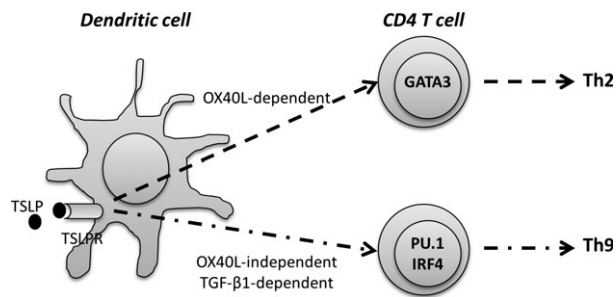


Figure 6 Proposed scheme for the role of thymic stromal lymphopoietin (TSLP) in priming mDCs for CD4⁺ T-cell activation in allergic asthma. TSLP signaling through mDCs can lead to the development of Th2 cells via OX40L induction, while it also promotes the development of Th9 cells through an OX40L-independent pathway requiring TGF- β 1.

induction by TSLP through mDCs are shown in Fig. 6, integrating that the OX40L-independent mechanism used by TSLP to promote Th9 responses relies on TGF- β , a key factor for reprogramming of Th2 cells into IL-9-producing Th cells (28).

The therapeutic effects of targeting the TSLP/TSLPR axis have been assessed in animal models of asthma. Zhang and colleagues reported reduced lung Th2 inflammatory responses to ovalbumin in mice treated by a soluble TSLP antagonist (TSLPR-Fc), which induced a downregulation of CD40, CD80, CD86, and TSLPR on DCs and abrogated the induction of Th2 responses by allergen-pulsed DCs (26). In addition to the inhibition of Th2 inflammation, another study showed that TSLP neutralization reduced goblet cell hyperplasia, collagen deposition, and TGF- β production (29). Treatment with anti-TSLPR mAb in a monkey model of asthma resulted, after 6 weeks of treatment, in decreased allergic inflammation and inhibition of expression of TSLP-related genes in the lung (30). Altogether, these studies and ours provide substantial evidence that targeting TSLP/TSLPR axis may effectively dampen salient features of Th2-related AA. In addition, based on recent evidence (11) and our present data, it will be interesting to evaluate Th9 responses upon the blockade of TSLP. It has already been described that IL-9-positive T cells are elevated in BAL from allergic patients after challenge (31). Another group showed increased numbers of blood Th9 cells in allergic patients as compared to controls (32). These data suggest a role for IL-9 in human asthma. However, the precise contribution of IL-9 to the pathogenesis of human asthma remains uncertain, as well as the dependency of the IL-9 pathway on TSLP.

Although our data strengthen the importance of the TSLP pathway in human asthma, our study presents with some limitations. First, the number of subjects was substantial but limited, due to requirements for extensive in-depth analyses (blood DCs) or for invasive sampling (endobronchial biopsies). Second, functional experiments were only performed on blood DCs and not on lung DCs. Albeit a continuum could be suspected, it remains unknown whether local reprogramming of DCs could affect TSLP reactivity. Third, the *in vitro*

model used in this study does not recapitulate the *in vivo* situation, considering the absence of key factors such as IL-33 (33) or IL-25 (6, 34) secreted by epithelial cells. Finally, the fact that the number of mDCs in bronchial biopsies was not increased in our study, in contrast to a previous one (35), could relate to the effect of treatment by inhaled steroids. Nevertheless, even considering this therapy, TSLPR expression remained increased in bronchial mDCs from these patients.

In conclusion, we report an increased expression of TSLPR in blood and bronchial mDCs from patients with AA and show for the first time that blood mDCs from these patients are consequently imprinted to drive Th2- and Th9-cell activation upon TSLP stimulation. We also show that this novel TSLP/mDC/Th9 axis does not rely on OX40L, in contrast to the corresponding Th2 axis, but rather depends on TGF- β . This study reinforces the knowledge about TSLP in the pathogenesis of AA and further suggests the interest of targeting this pathway in human asthma.

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Author contributions

AF designed and performed the experiments and wrote the manuscript. CS helped with experiments and revised the manuscript. DG and PC provided bronchial biopsies, analyzed patient characteristics, and revised the manuscript. JVS provided IL-9 reagents and helped with IL-9-related experiments. CP supervised the study and revised the manuscript.

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. (A) mDC gating. After positive selection with anti-CD1c microbeads, cells co-express CD11c, CD1c, and HLA-DR and do not express lineage 1 antigens (CD3, 14, 16, 19, 20, and 56). Hematoxylin–eosin staining after cyto-spin shows homogeneous cell population (scale bar, 50 μ m). (B) CD80 and CD86 expression on mDCs from allergic asthma patients and controls.

Figure S2. (A) CD4⁺ T-cell isolation, showing a purity rate >95% based on the expression of CD3 and CD4. (B) Changes in the proportion of CD45RA, CD45RO, and

CD25 expression in T cells stimulated by mDCs in the allo-MLR model. (C) Evolution of the proportion of positive cells for CD45RA, CD45RO, and CD25 during the MLR. The number of CD45RA⁺ naïve T cells decreased, while the number of CD45RO⁺ and activated CD25⁺ T cells increased upon the MLR. T cells alone do not upregulate CD45RO or CD25 over time. Data are representative of 3 independent experiments.

Table S1. Characteristics of study subjects for blood mDCs.

Table S2. Characteristics of study subjects for bronchial biopsies.

Data S1. Material and methods.

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Table S1: Characteristics of study subjects for blood mDCs

	Allergic asthma	Controls	P value
N subjects	18	15	
Sex ratio (M/F)	11/7	10/5	0.76
Age, years	40 (29-58)	56 (33-65)	0.23
FEV1, % predicted[†]	76.5 (67.7-89.1)	Nd	
FeNO, ppb[†]	64 (30-98)	Nd	
Daily ICS dose, µg eq. BDP	600 (600-1,152)	0	<0.0001

FEV1: forced expired volume in 1 second; **FeNO**: exhaled fraction of nitric oxide; **ICS**: inhaled corticosteroids; **BDP**: equivalent dose of Beclomethasone dipropionate.

Data are median and interquartile range.

Allergic status was defined according to a positive skin-prick test response > 5mm above diluent control and serum specific IgE > 0.35 kU/L (ImmunoCAP100, Phadia, Uppsala, Sweden) to at least *Dermatophagoides pteronyssinus* (Der p) allergen.

All asthma patients were treated with inhaled corticosteroids, but none with systemic steroids, anti-IgE or allergen immunotherapy.

[†]: the last available value at the time of enrollment was collected

Table S2: Characteristics of study subjects for bronchial biopsies

	Mild/moderate asthma	Severe asthma	Controls	P value
N subjects	6	9	4	
Age, years	48.5 (38-66)	55 (45.5-69.5)	30.5 (21.25-51.75)	0.12
Sex, M/F	2/4	4/5	3/1	0.44
FEV1, % predicted	99 (83.75-100) [†]	72 (65.5-77.5)	107.4 (101.7-108)	0.0009
ICS, N (% of total)	4 (67%) [†]	9 (100%)	0 (0%)	0.0001
Oral corticosteroids, N (% of total)	0 (0%)	5 (55%)	0 (0%)	0.03

FEV1: forced expired volume in 1 second; **ICS:** inhaled corticosteroids

All data are shown as median and interquartile range.

[†]: there was no information available about ICS use in two patients.

Supporting information

Myeloid dendritic cells are primed in allergic asthma for thymic stromal lymphopoietin-mediated induction of Th2 and Th9 responses

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Material and methods

Myeloid dendritic cell purification, culture and stimulation

Subjects underwent leukapheresis to obtain a buffy coat, and peripheral blood mononuclear cells (PBMC) were obtained after centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). Type 1 mDCs were isolated using immunomagnetic separation (MACS, Miltenyi Biotecs, Bergisch Gladbach, Germany). After B cell depletion with anti-CD19 microbeads, mDC were positively selected with anti-CD1c (BDCA 1) microbeads.

CD4⁺ T cells were purified from PBMC of 5 non-allergic, non-asthmatic donors using CD4⁺ T cells selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺ T cells were negatively selected using a cocktail of biotin-conjugated monoclonal antibodies coupled with anti-biotin microbeads.

For phenotyping, DCs were cultured overnight in 48 wells plates (500,000 cells/well at 1 million cells/mL) in RPMI-1640 medium supplemented with 10% of fetal calf serum, Streptomycin (100U/mL), Penicillin (100U/mL) and *L*-glutamine at a 2 mM concentration (Lonza, Verviers, Belgium), further referred as to complete RPMI (cRPMI). DCs were cultured either with or without TLR4 stimulation by LPS at 1 µg/mL (Sigma Aldrich, Saint-Louis, MO, USA).

For functional experiments, mDCs were cultured 48 hours in the presence of human TSLP (Miltenyi Biotec, Bergisch Gladbach, Germany), Der p extract (Stallergenes, Antony, France), LPS (Sigma Aldrich, Saint-Louis, MO, USA) or a combination. Blocking antibodies to OX40L (clone 159403, R&D, Minneapolis,

MN USA), IL-2 (clone AB12-3G4, eBioscience, San Diego, CA, USA), TGF- β 1 (clone 13A1, from co-author JVS) and control mouse IgG (eBioscience, San Diego, CA, USA) were used for neutralization experiments.

For T-cell assays, resting or stimulated mDCs were washed and cultured with allogeneic CD4⁺ T cells in 96 well plates at a ratio of 1:5 (40,000 mDCs for 200,000 T cells) for 5 days in cRPMI supplemented with 100 nM β -mercaptoethanol. T cells were analyzed after co-culture with pulsed mDCs, for cytokine production, either by ELISA or intracellular staining, or for lineage transcription factors of effector Th cells.

Immunophenotyping of dendritic cells and T cells

The following mAbs were used for flow cytometry: FITC-conjugated Abs to Lin 1 (CD3, 14, 16, 19, 20, 56) (clones NCAM16.2, M ϕ P9, L27, SJ25C1, 3G8, SK7), CD4 (clone RPA-T4), isotype-matched control IgG (clone MOPC-21, BD Pharmingen, San Diego, CA, USA), CD45RA (clone F8-11-13) and CD45RO (clone UCHL1, ImmunoTools, Friesoythe, Germany); PE-conjugated Ab to OX40L (clone ik-1), isotype matched control (clone MOPC-21, BD Pharmingen, San Diego, CA, USA), ICOSL (clone MIH12), IRF4 (clone 3E4), TSLPR (clone 1A6, eBioscience, San Diego, CA, USA), CD80 (clone L307.4), CD86 (clone 2331 -FUN-1-), and PU.1 (polyclonal, R&D, Minneapolis, MN USA) ; PerCP-conjugated Ab to HLA-DR (clone G46-6) and isotype-matched control IgG (clone X39, BD Pharmingen, San Diego, CA, USA); APC-conjugated Ab to CD1c (clone L161, eBioscience, San Diego, CA, USA), CD11c (clone B-ly6), CD25 (clone M-A251) and isotype-matched control IgG (clone MOPC-21, BD Pharmingen, San Diego, CA, USA); PE-Cy7-conjugated Ab to CD3 (clone HIT3a, Biolegend, San Diego, CA, USA), GATA3 (clone L50-823) and isotype-matched control IgG (clone MOPC-21, BD Pharmingen, San Diego, CA, USA); PerCP-Cy5-conjugated Ab to T-bet (clone eBio4B10) and isotype control (eBioscience, San Diego, CA, USA).

For immunostaining 5-10 x 10⁵ cells per sample were saturated with a solution of PBS-20% human serum for 30 minutes, to block Fc-receptors. After wash, cells were stained with fluorochrome-labelled

antibodies (at concentrations according to manufacturer's guidelines) for 45 minutes at 4°C, then fixed with 0.625 % formaldehyde. For intracellular staining, intracellular staining kit (eBiosciences, San Diego, CA, USA) was used. After surface staining for CD4 and CD3 at 4°C, T cells were washed and incubated in permeabilization buffer for 30 minutes before intracellular staining for GATA3 and fixation. Cell viability was assessed following propidium iodide staining (BD Pharmingen, San Diego, CA, USA) and was > 90% after 24hrs and >85% after 48 hrs, with or without LPS activation.

Flow cytometry analysis was performed using a FACS Canto™ II (BD Biosciences, San Diego, CA, USA). At least 10,000 events were read and results were analyzed with Flowjo software v7.6.5 (Tree Star, Ashland, OR, USA).

Immunoassays for cytokines

Cytokine levels were evaluated in culture supernatants by sandwich ELISA, using paired antibodies for detection of IL-5 (MAB405 / BAM6051), IL-13 (MAB213 / BAF213) (all from R&D, Minneapolis, MN USA), IL-9 (clones MH9A4/MH9A3, from co-author JVS), IFN-γ (MAB2852/BAF285) and IL-10 (BD554705 / BD554499, BD Pharmingen, San Diego, CA, USA). Detection limits were 8 pg/mL for IL-9 and 16 pg/mL for IL-13, IFN- γ, IL-10, and IL-5.

96 wells plates were coated with capture antibody overnight and then saturated for 1h with PBS containing 1% bovine serum albumin (BSA). After 3 washes with PBS-Tween 20 0.05%, 50 µL of each sample were incubated for 2 hours at room temperature. Biotinylated antibodies were added and incubated for 1 hr, and reaction was revealed by adding 50 µL of 0.01% streptavidin conjugated with horseradish peroxidase (BD Pharmingen, San Diego, CA, USA) for 30 minutes, followed by TMB (TetraMethylBenzidine, Thermo fisher, Meridian, Rockford, USA). After stopping the reaction with 100µl of 1.8M H₂SO₄/well, absorbance was read at 450 nm, using an ELISA microreader (Bio-Rad, Model 3550, Hercules, CA, USA). Concentrations were expressed in pg or ng/mL as referred to recombinant standards.

Immunohistochemistry for TSLPR and CD1c

Sections of endobronchial biopsies were first incubated with 0.3% H₂O₂ for 30 minutes to block endogenous peroxidases, and after washing incubated with 0.001% avidin and 0.001% biotin sequentially for 10 minutes respectively. After each step, sections were washed with PBS. Sections were then incubated with TBS-2%BSA for 30 min. Goat anti-human TSLPR antibody in 1%BSA (5 µg/mL, 1:40 dilution, AF981, R&D, Minneapolis, MN USA) or isotypic control were applied overnight at 4°C. After 3 washes (Tween 0.5% in TBS, pH 7.4), secondary antibody (rabbit anti-goat IgG Biotin conjugate, Sigma, B-7024, 1:3000 dilution) in 2% milk was applied for 30 minutes. Then streptavidin-Horseradish peroxidase (1:500; BD pharmingen, Erembodegem, Belgium) in 1%BSA-TBS was applied for 30 min, and reaction was developed using diaminobenzidine (DAB) for 5 min. Sections were rinsed in distilled water and again blocked with 2% milk for 30 minutes at room temperature. Anti-CD1c antibody (mouse anti-human CD1c mAb, clone 2F4, Abcam, Cambridge, UK) in 1%BSA TBS or isotype control were applied overnight at 4°C, followed by biotinylated rabbit anti-mouse IgG antibody (Sigma, B-7024, 1:1000 dilution) in 1%BSA for 30 minutes. Then streptavidin-Horseradish Alkaline Phosphatase (1:1000; BD pharmingen, Erembodegem, Belgium) in 1%BSA-TBS was applied for 30 min, before development with Fast-Red (Sigma Chemical Co., St Louis, MO, USA) and counterstaining with hematoxylin. Sections were observed under optical microscope (Zeiss Axiocam) and images were acquired using Leica SCN400 slide scanner (Meyer Instruments, Houston, TX, USA), analyzed with SlidePath software (Leica, Soms, Germany). Immunostained sections were read by one investigator (AF) blind to the clinical data, and results were expressed as DC numbers per mm² of bronchial tissue.

Supplementary word count: 1115

Figure legends

Figure S1: A, mDC gating. After positive selection with anti-CD1c microbeads, cells co-express CD11c, CD1c and HLA-DR and do not express lineage 1 antigens (CD3, 14, 16, 19, 20 and 56). Hematoxylin-eosin staining after cytopspin shows homogeneous cell population (scale bar, 50 μ m). **B**, CD80 and CD86 expression on mDCs from allergic asthma patients and controls.

Figure S2: A: CD4⁺ T cell isolation, showing a purity rate >95% based on the expression of CD3 and CD4. **B**: changes in the proportion of CD45RA, CD45RO and CD25 expression in T cells stimulated by mDCs in the alloMLR model. **C**: evolution of the proportion of positive cells for CD45RA, CD45RO and CD25 during the MLR. The number of CD45RA⁺ naïve T cells decreased, while the number of CD45RO⁺ and activated CD25⁺ T cells increased upon the MLR. T cells alone do not upregulate CD45RO or CD25 over time. Data are representative of 3 independent experiments.