

Research Article

STAT6 inhibitory peptide reduces dendritic cell migration to the lymph nodes to control Th2 adaptive immunity in the mouse lung

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Type 2 immunity in the lung is promoted through the release of innate cytokines, including TSLP, from lung structural cells. These cytokines drive Type 2 immunity in part through upregulation of OX40L on dendritic cells (DCs). DCs expressing OX40L are potent inducers of Th2 differentiation. We have shown previously that STAT6 inhibitory peptide (STAT6-IP), a cell penetrating peptide designed to inhibit the STAT6 transcription factor, reduces the induction of Th2 adaptive immunity in murine models of respiratory syncytial virus infection. Here we show that intranasal administration of STAT6-IP at the time of antigen priming with ovalbumin (OVA), in conjunction with the Nod2 agonist, MDP, reduced frequencies of CD11b⁺ lung DCs expressing OX40L. Consistent with these reductions, fewer activated DCs were localized to the lung draining lymph nodes in STAT6-IP-treated mice. Upon OVA challenge four weeks later, mice treated with STAT6-IP at the time of OVA/MDP priming did not develop airway hyperresponsiveness (AHR) and had reduced influx of eosinophils into the airways, mucus production, and serum OVA-specific IgE levels. Our findings provide evidence that the long-lasting inhibitory effects of STAT6-IP are due in part to inhibition of DC responses that drive maladaptive Th2 adaptive immunity and allergic airways disease.

Keywords: Airway hyperresponsiveness · Asthma · Dendritic cells · STAT6-IP · Type 2 adaptive immunity



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Allergic asthma is a chronic inflammatory disease of the airways caused by maladaptive Type 2-biased immune responses to environmental allergens [1, 2]. Key features of allergic asthma are airway hyperresponsiveness (AHR), mucus production, airway

remodeling and obstruction associated with increases in lung and circulating eosinophils, IgE, and Type 2 cytokines, including IL-4, IL-5, and IL-13 [3–5]. Corticosteroids and β -agonists are the most effective drugs for the treatment of asthma in both children and adults [6]. However, they target only the symptoms, but not the cause of disease. Moreover, up to 60% of asthmatics respond poorly to corticosteroids, highlighting the need for more effective therapies [7–9]. There is renewed interest in developing therapeutic strategies that target airway inflammatory responses, including Type 2 cytokines and their receptors, following

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positive outcomes from a number of human studies and clinical trials [10–12]. Understanding mechanisms that initiate airway inflammatory responses prior to symptom onset could provide targets for development of additional therapeutics with the ability to diminish asthma incidence.

At mucosal surfaces, structural cells, including epithelial cells, promote Type 2 inflammatory responses through the release of innate cytokines, including IL-25, thymic stromal lymphopoietin (TSLP), and IL-33 [13, 14]. Data from murine asthma models indicate that TSLP and IL-33, released after activation of pattern recognition receptors, including TLR4 and nucleotide-binding oligomerization domain-containing protein (NOD) receptors, induce responses in a variety of innate cells in the airways to promote Type 2 immunity. While recent interest has focused on the ability of these cytokines (in particular IL-33) to induce production of IL-13 (and IL-5) from lung group 2 innate lymphoid cells (ILC2s) [15, 16], TSLP and/or IL-33 induce responses in a number of innate lung cells with the ability to promote Type 2 immunity, including macrophages [17–19], NKT cells [20], eosinophils [21], and dendritic cells (DCs) [13, 22].

TSLP, IL-33, and IL-25 all have the ability to upregulate expression of the costimulatory molecule, OX40 ligand (OX40L; CD252), on B cells and DCs [22, 23], and OX40L-expressing DCs are potent inducers of Th2 differentiation [24–29]. The ability of OX40L-expressing DCs to induce Th2 adaptive immunity is likely mediated, at least in part, through induction of IL-4 production from target CD4⁺ T cells, coupled with suppression of IL-10 [26, 30]. Activation of the OX40L target (OX40) on CD4⁺ T cells promotes cell survival through increased expression of the anti-apoptotic protein, B-cell lymphoma 2 (Bcl2) [31], and has been linked to Th2 effector memory responses in the lung [32].

Accumulating evidence suggests that STAT6 activation in DCs may promote Type 2 inflammation in the lung. DCs express STAT6 as well as receptors for IL-4 and IL-13 and thus have the ability to respond directly to these cytokines [33–36]. Data from Liu and colleagues provided evidence that TSLP could induce STAT6 activation in a manner *independent* of IL-4 receptor signaling in human myeloid DCs, suggesting that TSLP could *directly* induce STAT6 activation in DCs to promote Type 2 immunity [33]. Luster and colleagues highlighted the importance of STAT6 in CD11b⁺ murine myeloid DCs in promoting Th2 cell and eosinophil recruitment into the airways via production of CCL17, CCL22, and CCL24 [37]. Moreover, compelling data from Takei and colleagues provided evidence that IL-33-induced IL-13 production from ILC2s promotes DC migration to the mediastinal lymph node and Th2 priming, though a precise role for DC-specific STAT6 activation was not assessed in this study [16]. Together, these data suggest that STAT6 activation, specifically in DCs, has the ability to promote both the induction of Th2 differentiation in the lymph node as well as the subsequent recruitment of Th2 cells (and eosinophils) to the airways upon allergen challenge.

We have developed and characterized a cell penetrating STAT6 inhibitory peptide, STAT6-IP [38–40]. This peptide is comprised of a protein transduction domain (PTD4), for cellular uptake, coupled to 8 amino acids surrounding phosphotyrosine (*Y) 641 of

murine STAT6, and was designed to bind to the SH2 domain of STAT6, acting in a dominant negative manner to prevent dimerization of STAT6. A negative control, STAT6 control peptide (STAT6-CP), in which the *Y was replaced with phenylalanine (F), has also been used [38–40]. Intranasal delivery of STAT6-IP to sensitized mice at the time of antigen/allergen challenge reduces airway inflammatory responses (AHR, bronchoalveolar lavage [BAL] fluid eosinophil numbers, mucus production) in both acute and chronic models of allergic airways disease [38, 41]. Intranasal STAT6-IP delivery also reduces Type 2-biased airway inflammatory responses in respiratory syncytial virus (RSV) infection models [39, 40].

Here we examined the ability of STAT6-IP to reduce Th2 priming in the lung induced by local delivery of the model antigen, ovalbumin (OVA), and the Th2 skewing Nod2 agonist, muramyl dipeptide (MDP) [23, 27]. We show that STAT6-IP reduced frequencies of OX40L-expressing DCs in the lung; in addition, fewer activated DCs were present in the lung draining mediastinal lymph nodes (MLN) in OVA/MDP-primed mice exposed to STAT6-IP. Inhibition of DC responses in these mice had long-lasting effects: STAT6-IP treated mice challenged with OVA 28 days later did not develop AHR and had reduced airway inflammatory responses, including eosinophil recruitment, mucus production and OVA-specific IgE production. Collectively, our data show that STAT6-IP delivery to the lung effectively disrupts DC migration to the MLN and reduces Th2 adaptive immunity responsible for pathogenesis in allergic airways disease.

Results

STAT6-IP reduces OVA/MDP-induced OX40L-expressing DCs in the lungs

Croft and coworkers previously showed that C57Bl/6 mice treated intranasally with OVA in the presence of MDP, a Nod2 agonist, had increased TSLP and OX40L mRNA and protein levels in the lungs, compared to mice treated with OVA alone [23]. We examined if OVA/MDP increased OX40L in OVA/MDP-treated Balb/c mice and whether STAT6-IP had the ability to modulate this response. Mice were treated intranasally with OVA only (OVA group), or in the presence of MDP (OVA/MDP group), alone or with STAT6-IP or STAT6-CP as shown in Fig. 1A. OX40L mRNA levels were increased in the lungs of mice treated with OVA/MDP and this increase was significantly reduced by STAT6-IP (Fig. 1B); STAT6-CP moderately reduced OX40L mRNA levels but this difference was not significant (Fig. 1B). Levels of TSLP mRNA in OVA/MDP-treated mice were not affected by STAT6-IP or STAT6-CP treatment (data not shown). We next examined how STAT6-IP affected OX40L expressing CD11b⁺ lung DCs in OVA/MDP-treated mice following the gating strategy presented in Fig. 1C. We also examined effects of STAT6-IP (and STAT6-CP) on CD86- and MHCII-expressing lung DCs; in the case of MHCII, we focused primarily on DCs expressing the highest levels of MHCII (MHCII^{hi} - Supporting Information Fig. 1A). Frequencies of lung DCs expressing

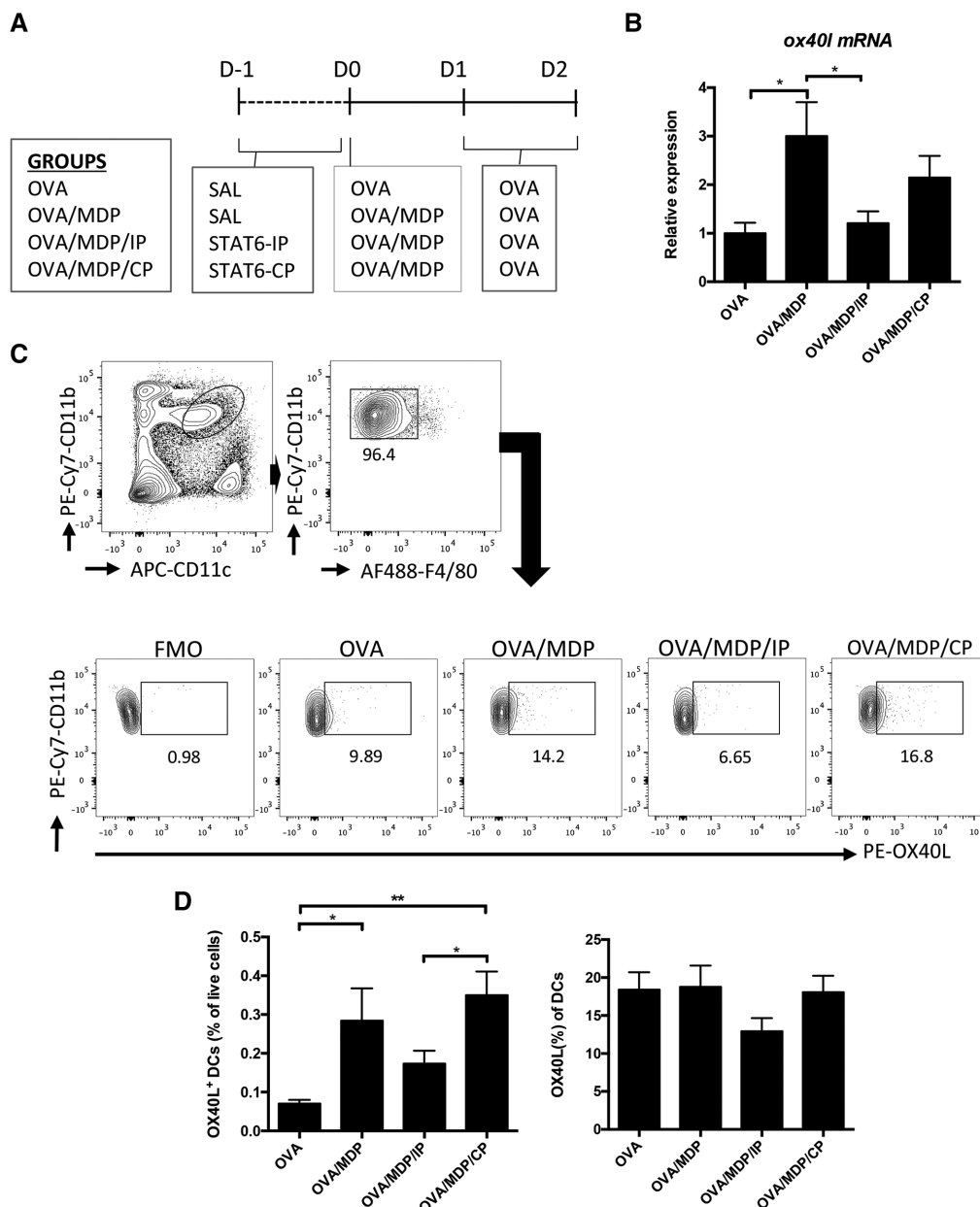


Figure 1. STAT6-IP decreases OX40L mRNA levels and surface expression on DCs in OVA/MDP treated mice. (A) Mice were treated intranasally with OVA each day for three consecutive days, either alone or with MDP on the first day. Mice were treated intranasally with saline, STAT6-IP, or STAT6-CP one day before and 1h before the first OVA/MDP priming. Mice were sacrificed 3h after the last exposure. (B) OX40L mRNA levels, normalized to GAPDH, and relative to levels in OVA-treated mice. (C, D) Total lung cells were stained as indicated in the *Materials and Methods* and analyzed by flow cytometry. Cells were gated first for viability and singlets (as in Supporting Information Fig. S1), and then as CD11c⁺CD11b⁺F4/80⁺ DCs, followed by gating for expression of OX40L. Representative log-scale flow cytometry plots are shown in C and frequencies of CD11b⁺OX40L⁺ DCs in the lung (left-hand panel) or frequencies of OX40L⁺ DCs within the CD11b⁺ DC population (right-hand panel) are shown in D. (B and D) Data are presented as the mean +SEM pooled from three independent experiments with three mice per group in each experiment in B and from six independent experiments, with three to five mice per group in each experiment in D. Outcomes were assessed by one-way ANOVA, followed by Newman–Keuls multiple comparison test. * $p < 0.05$, ** $p < 0.01$.

OX40L were significantly increased in mice treated with OVA/MDP either alone or in the presence of STAT6-CP. STAT6-IP effectively reduced these increases (Fig. 1D, left-hand panel). There was also a trend for STAT6-IP to reduce the frequency of both OX40L (Fig. 1D, right-hand panel) and MHCII^{hi} (Supporting Information Fig. 1B, right-hand panel) specifically on lung CD11b⁺

DCs, though these differences did not reach significance. Neither MDP nor STAT6-IP modulated CD86-expressing DC populations in the lung (Supporting Information Fig. 1C). Together, these data demonstrate that STAT6-IP, but not STAT6-CP, reduced CD11b⁺ DCs expressing OX40L and, possibly, MHCII in OVA/MDP-treated mice.

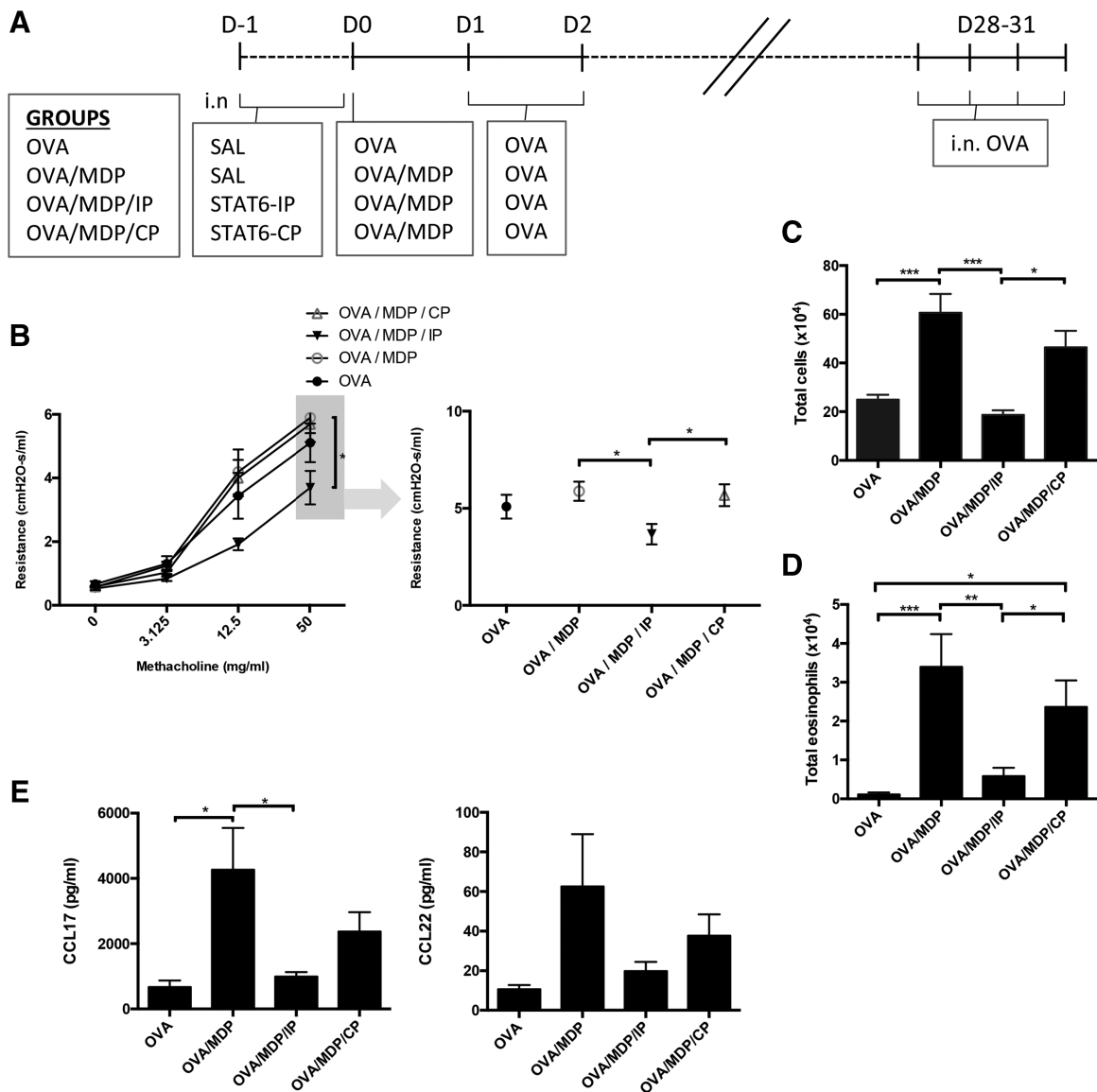


Figure 2. STAT6-IP treatment at OVA/MDP priming reduces airway inflammatory responses. (A) Mice were treated with OVA, MDP and STAT6-IP or STAT6-CP as described in Fig. 1. After a minimum of 28 days, mice were challenged with OVA once per day for each of 4 days and sacrificed 48 h later. (B) Lung function was assessed with the flexiVent small animal ventilator and is presented as lung resistance: dose response (left) and 50 mg/mL dose (right). BAL fluid was collected for quantification of (C) total cell numbers, (D) eosinophils, (E) CCL17 and CCL22. (B–E) Data are presented as the mean $\pm$ SEM pooled from three independent experiments with three to four mice per group in each experiment for B and from five independent experiments with three to five mice per group in each experiment for C–E. Outcomes were assessed by one-way ANOVA, followed by Newman–Keuls multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

STAT6-IP reduces allergic airway inflammatory responses induced upon OVA challenge

Neutralizing OX40L at the time of antigen priming reduces Th2 adaptive immunity in allergy and RSV infection models [23, 42–45]. We hypothesized that the early modulation of DCs by STAT6-IP would reduce Th2-dependent responses following OVA challenge. Thus, we examined airway inflammatory responses following OVA challenge in mice treated with OVA or OVA/MDP, alone or in the presence of STAT6-IP or STAT6-CP (Fig. 2A). Total lung resistance (Fig. 2B) and elastance

(Supporting Information Fig. 2A) were significantly reduced in mice treated with STAT6-IP at the time of OVA/MDP priming. Total cell numbers as well as eosinophils were increased in the BAL fluid of OVA/MDP treated mice and these were also significantly decreased in mice treated with STAT6-IP at the time of OVA/MDP priming (Fig. 2C, D). OVA/MDP induction and effects of STAT6-IP and STAT6-CP were more variable for other cells in the BAL fluid - macrophages, neutrophils and lymphocytes (Supporting Information Fig. 2B). STAT6-IP delivery at the time of OVA/MDP priming reduced BAL fluid levels of the Th2 chemokine CCL17 (TARC) (Fig. 2E). Another Th2 chemokine, CCL22 (MDC)

showed a similar profile of induction by OVA/MDP and inhibition by STAT6-IP though the differences did not reach statistical significance (Fig. 2E). Levels of CCL11, an eosinophil chemokine, were below the limit of detection (data not shown). Altogether, these data demonstrate that STAT6-IP delivery, selectively at the time of OVA/MDP-induced Th2 priming, reduced allergic airways disease induced upon OVA challenge 4 weeks later.

STAT6-IP reduces airway inflammation and goblet cell hypertrophy/metaplasia following OVA challenge

Consistent with the induction of AHR and eosinophil influx into the airways in OVA/MDP-primed mice following OVA challenge, these mice also had increased inflammatory cell infiltration (Fig. 3A) and mucus production (Fig. 3B) in the airways compared to OVA-treated control mice. These responses were reduced in mice treated with STAT6-IP but not STAT6-CP (Fig. 3A, B). Thus, STAT6-IP delivery at priming inhibited OVA/MDP-induced airway inflammation and goblet cell metaplasia upon OVA challenge.

STAT6-IP reduces OVA-specific IgE and CD4⁺ T cell-specific IL-4 production

The reductions in Th2-biased airway inflammatory responses in OVA-challenged mice treated with STAT6-IP at the time of priming suggested that STAT6-IP targeting of lung DCs to reduce OX40L could result in diminished Th2 differentiation [32]. Thus, we assessed if STAT6-IP modulated serum OVA-specific IgE levels as well as IL-4-producing CD4⁺ T cells in BAL fluid. OVA-specific IgE levels were increased in OVA/MDP-treated mice and this induction was significantly inhibited by STAT6-IP, but not by STAT6-CP, delivery at priming (Fig. 4A). To examine how STAT6-IP and STAT6-CP affected IL-4-producing BAL fluid CD4⁺ T cells, we used the gating strategy presented in Fig. 4B. While the total number of BAL fluid CD4⁺ T cells did not differ significantly between groups (Fig. 4C, left-hand panel), the number of IL-4-expressing CD4⁺ T cells was significantly reduced by STAT6-IP, compared to mice treated with OVA/MDP (Fig. 4C, right-hand panel). STAT6-CP-treated mice tended to have lower numbers of IL-4-producing T cells, though this difference was not significant. IL-13-expressing CD4⁺ T cells were very few in number, though each cell expressing IL-13 also expressed IL-4 (data not shown).

STAT6-IP reduces OVA/MDP-induced DC migration to the mediastinal lymph nodes

In order to more directly address DC-specific effects of STAT6-IP, bone marrow derived DCs (BMDCs) were cultured overnight with saline or TSLP, either alone or in the presence of STAT6-IP or STAT6-CP. We chose TSLP in these experiments (in place of MDP) based on data demonstrating that MDP upregulates OX40L on target DCs indirectly via TSLP production from stromal cells

[23, 27]. BMDCs were washed to remove TSLP and then loaded with OVA peptide and the ability of these cells to induce cytokine production from co-cultured naïve CD4⁺ T cells was assessed. While IL-4 producing CD4⁺ T cells were few in number and did not differ between groups (data not shown), the frequency of IL-13-expressing CD4⁺ T cells was modestly reduced by STAT6-IP, but not STAT6-CP (Supporting Information Fig. 3A&B). Though these data do not rule out indirect effects of STAT6-IP on DCs to control CD4⁺ T cell cytokine production in vivo, they do suggest that STAT6-IP could directly target DCs to reduce Th2 differentiation.

Recently, the ability of IL-13 to induce migration of DCs to the lung draining MLN has been implicated in Th2 differentiation in house dust mite and papain allergy models [15, 16]. Because IL-13 is a potent inducer of STAT6 activity, these data suggest that DC-specific STAT6 activity could be required for this response. Thus, we examined the ability of STAT6-IP to modulate OVA/MDP-induced DC migration to the lung MLN. The gating strategy to identify MHCII- and CD86-expressing DCs in the MLN is shown in Fig. 5A. As expected, the total number of cells in the MLN was increased in mice exposed to OVA/MDP (Fig. 5B). This increase was significantly reduced by STAT6-IP and unaffected by STAT6-CP (Fig. 5B). Moreover, the OVA/MDP-induced increases in the number of MHCII^{hi} DCs (Fig. 5C), as well as those expressing CD86 (Fig. 5D), in the MLN were also reduced by STAT6-IP and unaffected by STAT6-CP. The frequency of OX40L expressing DCs in the MLN was very low; these cells were subtly increased in OVA/MDP- (and OVA/MDP plus STAT6-CP) exposed mice and decreased in mice treated with STAT6-IP, though these changes did not reach statistical significance (data not shown). Together, these data suggest that STAT6-IP inhibits OVA/MDP-induced DC activation and migration to the MLN, the outcome of which is diminished Th2 priming and reduced allergic airway inflammatory responses following OVA challenge.

Discussion

A large literature describes activities of STAT6 in CD4⁺ T cells regulating gene expression and chromatin remodeling in Th2 differentiation [46]. STAT6 also regulates responses in a number of cells in the lung that participate in asthma pathogenesis [47]. That STAT6 signaling in structural cells of the airways is required for Th2 cell and eosinophil recruitment into the lungs was originally shown by Luster and colleagues who demonstrated that differentiated Th2 cells, when adoptively transferred into STAT6 knockout mice, were unable to migrate to the airways and unable to induce recruitment of host eosinophils [37, 48]. While epithelial cells were originally considered the main source of chemokines that promoted Th2-biased inflammation in this model [48], these authors subsequently showed that the cellular source of chemokines was actually a CD11b-expressing myeloid cell [37]. In our original STAT6-IP studies [38, 41], STAT6-IP delivered to sensitized mice at the time of OVA or ragweed challenge, reduced a number of allergic inflammatory outcomes in the lung. The target cells through which

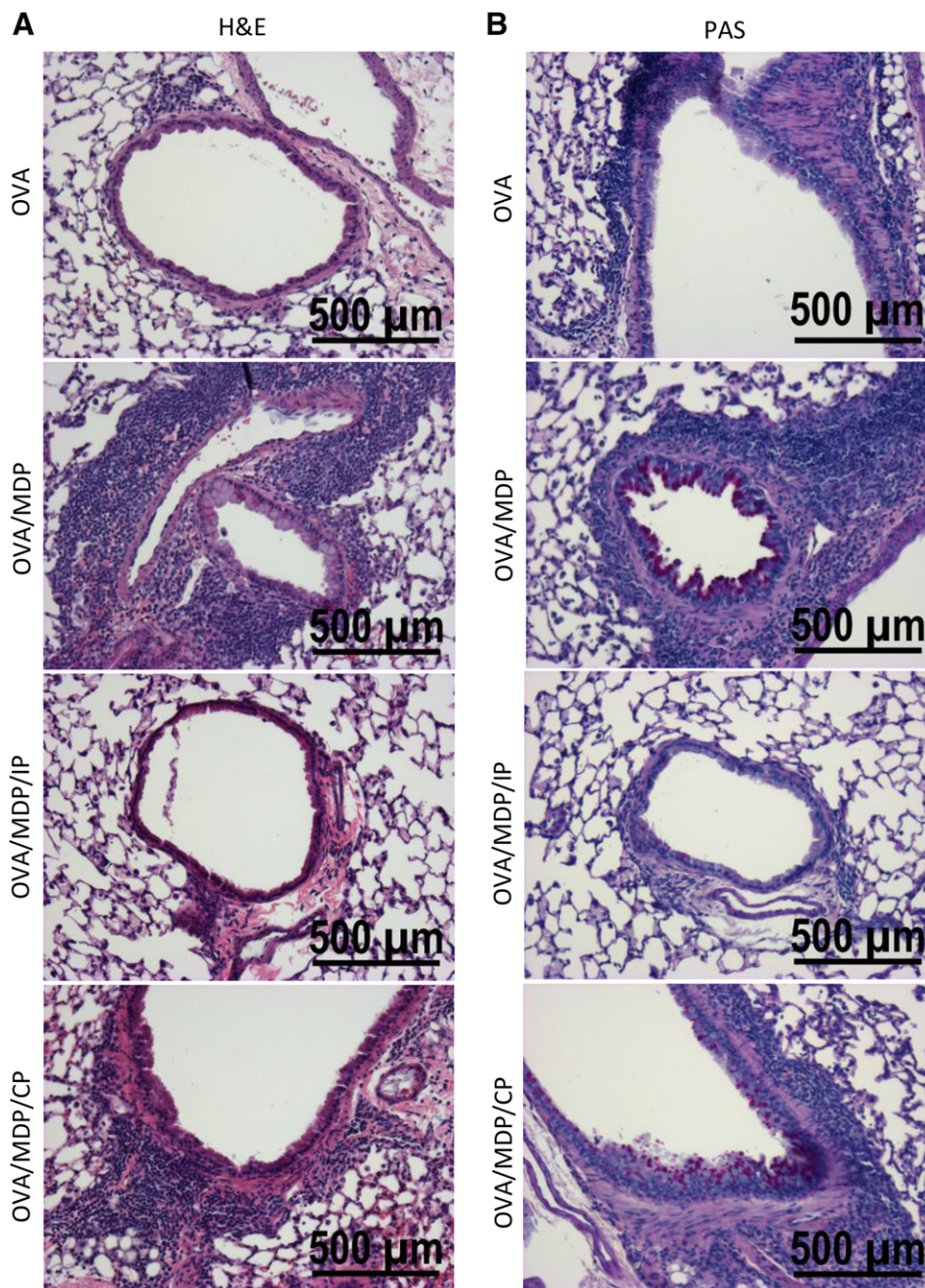


Figure 3. STAT6-IP treatment at OVA/MDP priming reduces lung inflammation and mucus production following OVA challenge. Mice were treated with OVA, MDP and STAT6-IP or STAT6-CP and challenged with OVA as described in Fig. 2. (A) H&E staining and (B) PAS staining on lung airways. Magnification $\times 10$. Images shown are from one experiment representative of 3 independent experiments ($n = 2$ mice per experiment).

STAT6-IP mediated its inhibitory activity were poorly defined. Our subsequent studies [39, 40] and those of Michael et al. [49] implicated DCs and macrophages in STAT6-IP-dependent inhibitory responses.

To more precisely examine effects of STAT6-IP in DCs in the lung, we developed a new model, related to a lung tolerance model originally described by Duan, et al., [23] where mice are treated with a purified preparation of OVA in the presence of a Nod2 agonist, MDP. These authors had shown that activation of Nod2 in the lung promotes Th2 differentiation and reduces Treg differentiation, thereby blocking the ability of OVA to induce tolerance. Induction of Th2 immunity was linked to TSLP pro-

duction, possibly from airway stromal cells that induced OX40L expression on DCs and B cells. While their model relied upon subsequent intraperitoneal OVA/alum sensitization to assess the effect of MDP delivery to the lung, we reasoned that OVA/MDP would be sufficient for Th2 priming, the outcome of which could be assessed simply by subsequent OVA challenge. Our model is also related to that of Lei et al., who showed that lung delivery of OVA with recombinant TSLP (bypassing induction by MDP) effectively promotes allergic airways disease upon subsequent OVA challenge [50].

Our data show that STAT6-IP effectively reduced OX40L upregulation at the mRNA level in the lung, and reduced OX40L

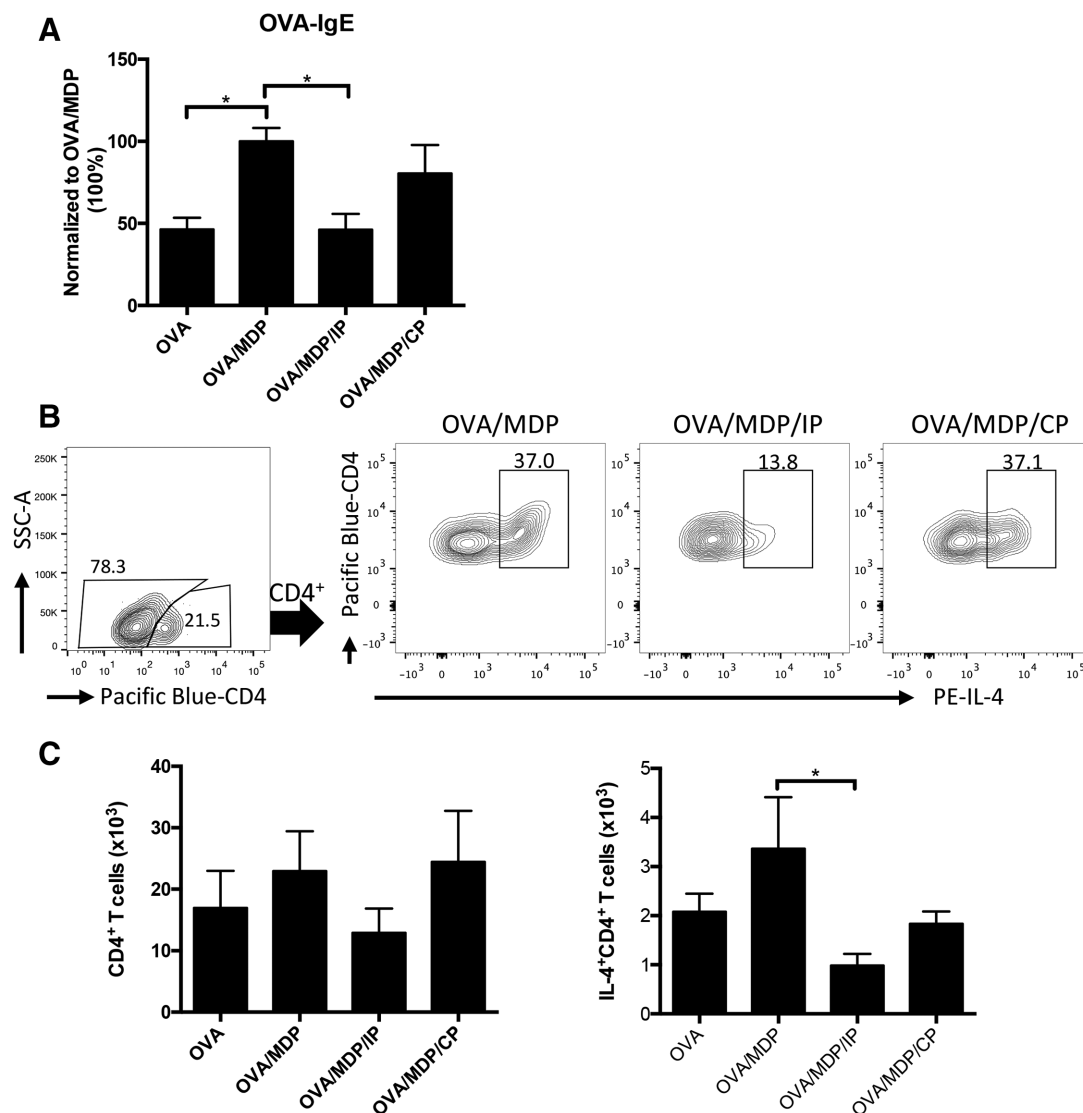


Figure 4. STAT6-IP treatment at OVA/MDP priming reduces OVA-specific IgE and CD4⁺ T cell-specific IL-4 production. (A) OVA-specific IgE levels in serum, normalized to OVA/MDP group, set at 100%. (B) Total BAL fluid cells were stained as indicated in the *Materials and Methods* and analyzed by flow cytometry. Cells were gated first for viability and singlets (as in Supporting Information Fig. 1), and then as CD4⁺ T cells, followed by gating for expression of IL-4. Representative log-scale flow cytometry plots are presented for IL-4 expressing CD4⁺ T cells. (C) Total number of BAL fluid CD4⁺ T cells (left-hand panel) or IL-4-expressing CD4⁺ T cells (right-hand panel). (A and C) Data are presented as the mean +SEM pooled from three independent experiments, with three to five mice per group in each experiment. Outcomes were assessed by one-way ANOVA, followed by Newman–Keuls multiple comparison test. * $p < 0.05$.

(and possibly MHCII) expressing lung CD11b⁺ DCs. STAT6-IP also reduced the ability of these DCs to migrate to the lung draining MLN where they would promote Th2 differentiation. While no differences were found in the lung, significant reductions in CD86-expressing DCs in the MLN in STAT6-IP-treated mice were found. Upon OVA challenge 4–5 weeks later, allergic airway inflammatory responses in STAT6-IP treated mice were diminished. AHR was not induced and mucus production, influx of eosinophils into the airways and OVA-specific IgE levels were all significantly reduced in mice that had been treated with STAT6-IP at the time of OVA priming. Our data agree with others showing reductions in the same parameters of allergic airways disease in mice lacking either OX40 [51] or OX40L [52, 53].

The precise mechanism by which STAT6-IP interfered with DC responses in the lung and MLN in OVA/MDP-treated mice is not clear. TSLP is a potent inducer of OX40L upregulation on DCs [22, 26] but any link between STAT6 activation by TSLP and subsequent OX40L upregulation does not appear to have been addressed. Data from Arima et al. showed that TSLP induced activation of STAT6 in human myeloid DCs in a manner that was *independent* of the IL-4 receptor [33]; however, we have been unable to replicate these data in murine cells (data not shown). While TSLP may not activate STAT6 directly, it does have the ability to induce production of both IL-4 and IL-13 [42, 54, 55], both of which could then activate STAT6 in DCs. In addition, the ability of STAT6 to promote DC activation is implicated in the work of

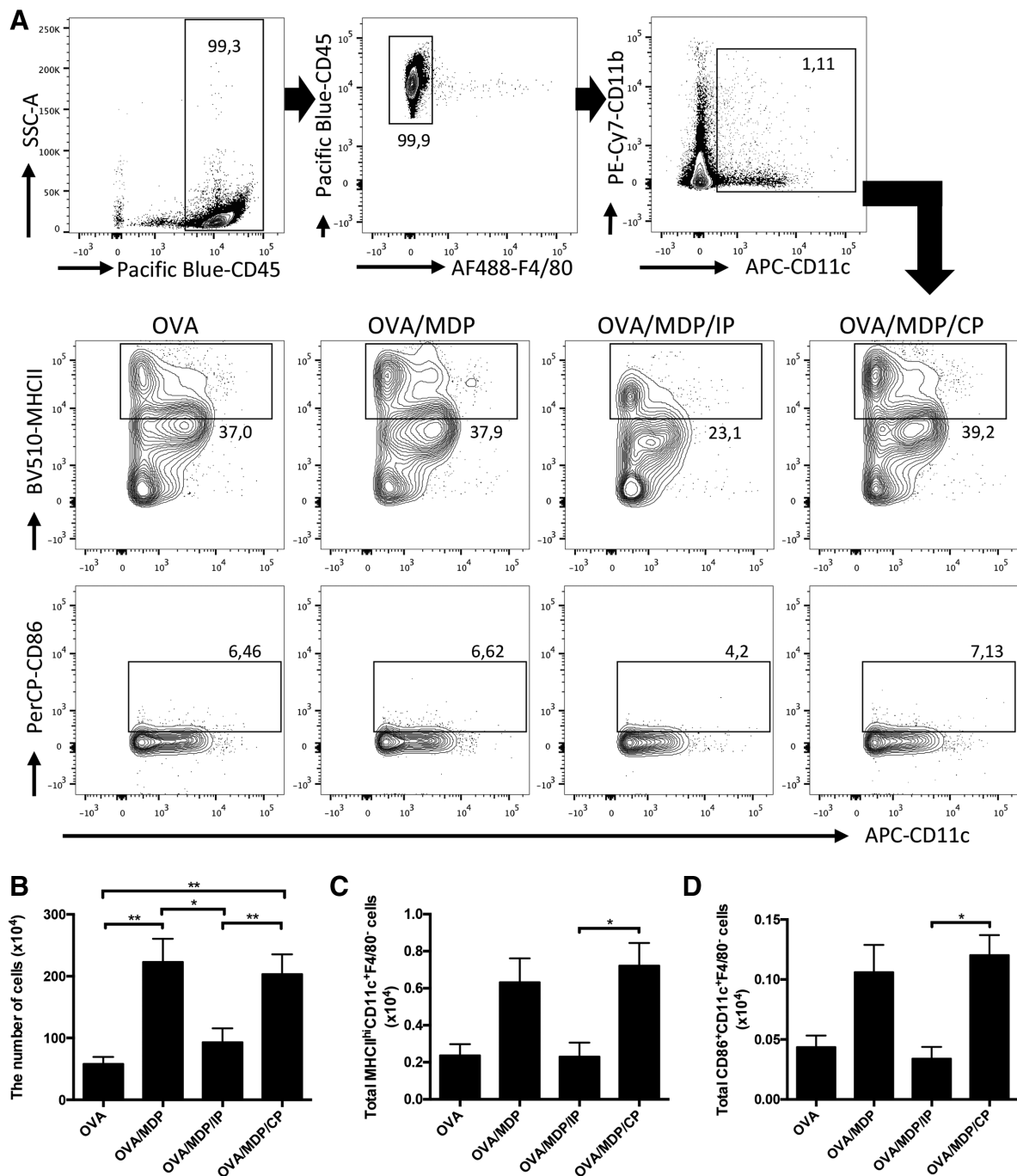


Figure 5. STAT6-IP reduces OVA/MDP-induced DC migration to the mediastinal lymph nodes. (A) Total LN cells were stained as indicated in the *Materials and Methods* and analyzed by flow cytometry. The cells were gated first on live cells and singlets (as in Supporting Information Fig. 1), and then gated on CD45⁺CD11c⁺F4/80⁻ DC population. Then, MHCII⁺ or CD86⁺ cells were gated from the DC population. (B) Total LN cells. (C) Total number of CD11c⁺MHCII⁺ cells. (D) Total number of CD11c⁺CD86⁺ cells. Representative log-scale flow cytometry plots are shown in A. (B–D) Data are presented as the mean + SEM from four to six mice per group (except for OVA, where $n = 3$) from one experiment, representative of three independent experiments. Outcomes were assessed by one-way ANOVA, followed by Newman-Keuls multiple comparison test. * $p < 0.05$. ** $p < 0.01$.

Halim et al., who showed that IL-13 produced by ILC2s promoted DC migration to the MLN, though these authors did not present changes in OX40L in this model. A role for IL-13 in upregulation of costimulatory molecules is, however, provided by data from Padilla et al., who showed that administration of recombinant

IL-13 to the lung increases MHCII and CD40 on lung CD11c⁺ cells in a manner that is completely dependent upon expression of the IL-4R α , one subunit of the cognate IL-13 receptor [56]. While STAT6-IP-dependent changes in MHCII^{hi}- or CD86-expressing DCs in the lung were quite modest (or absent) in mice treated

with OVA/MDP, these DCs (as well as those expressing CD40 [data not shown]) were significantly reduced in the MLN of STAT6-IP-treated mice. Unfortunately, it was not possible to reliably quantify OX40L-expressing DCs due to the small number of these cells in the MLN. Our data thus implicate MDP-induced IL-13 production in these increases in markers of DC activation. The precise molecular mechanism by which IL-13, STAT6, and STAT6-IP regulate DCs expressing OX40L and other co-stimulatory molecules in both the lung and MLN is an ongoing area of our research.

We also found that OVA-specific IgE levels were significantly reduced in STAT6-IP treated mice, suggesting that follicular helper T (T_{fh}) cells, which promote IgE production in response to allergen administration [57, 58], were reduced. These decreases could be due to STAT6-IP-dependent reductions in OX40L-expression, which promote T_{fh} responses and IgE production [59–61]. However, recent data from Kita and colleagues showed that OX40L was essential for Th2-dependent eosinophilic airway inflammation but dispensable for IgE production in an *Alternaria alternata* model [57], suggesting that these responses might be uncoupled. Whether the STAT6-IP-dependent reductions in OX40L (and MHCII) expression on DCs was responsible for diminished IgE levels in OVA/MDP-sensitized mice following OVA challenge warrants further investigation.

Lung DC subsets are categorized largely as cDCs (conventional DCs), moDCs (monocyte derived DCs), and pDCs (plasmacytoid DCs). Lung cDCs are further divided into cDC1 (CD103⁺ cDCs) and cDC2 (CD11b⁺ cDCs) subsets. Medoff et al. have shown that CD11b⁺ myeloid cells promote Th2 and eosinophil recruitment to the lung in a STAT6-dependent manner [37]. Moreover, similar to our data, and that of others [23] showing OX40L on CD11b⁺ DCs in the lungs of OVA/MDP treated mice, OX40L may be preferentially expressed on CD11b⁺ DCs, whether they are cDC2 or moDCs [15, 44]. Our in vitro data from BMDCs showed only subtle changes in OX40L expression induced by various mediators (e.g. OVA/TSLP), and its inhibition by STAT6-IP (data not shown). Moreover, while we found that STAT6-IP inhibited the ability of OVA/TSLP-stimulated BMDCs to increase IL-13 expression in co-cultured naïve CD4⁺ T cells, these in vitro responses were quite modest. The difference between our in vivo and in vitro findings may be due in part to the fact that the in vivo responses are selectively mediated by CD11b⁺ cDC, while GM-CSF-derived BMDCs, which we used in our in vitro studies, are a more heterogeneous population, the majority of which appear to be most closely related to CD11b⁺ moDC [62]. Of course, the in vitro BMDC:CD4⁺ T cell co-culture system is static and cannot be used to study DC migration. In addition, OX40L is also expressed by macrophages [63] and B cells [64] and these cells may also promote allergic inflammatory responses in the lung and they may also be targets of STAT6-IP. In fact, an ongoing area of our research is to characterize STAT6-IP effects in macrophages, based on our published data [39, 40]. STAT6-IP targeting of multiple cell types may contribute to the more robust inhibitory responses found in vivo compared to the more reductionist in vitro BMDC:CD4⁺ T cell co-culture experiments.

Our data suggest that STAT6-IP inhibited OVA/MDP-induced DC migration to the lung MLN. Total MLN cell numbers increased in OVA/MDP treated mice compared to control OVA-treated mice and this increase was selectively reduced by STAT6-IP. Consistent with effects on lung DCs, STAT6-IP, but not STAT6-CP, inhibited the increase in total number of DCs in the MLN of OVA/MDP treated mice. We have also assessed expression of co-stimulatory molecules on DCs localized to the MLN and, as discussed above, found modest decreases in OX40L as well as other co-stimulatory molecules, in those from STAT6-IP treated mice. Our data showing STAT6-IP disrupts the ability of OVA/MDP to induce DC migration to the MLN support findings from Halim et al., who showed that IL-13 induced migration DCs to the MLN to promote Th2 differentiation [16].

Overall, our data demonstrate that inhibitory activity of STAT6-IP is mediated at least in part by reduced activation and migration of lung DCs to the lung draining lymph nodes, effectively reducing Th2 priming and subsequent allergic airways disease upon secondary exposure to antigen, even many weeks later.

Materials and methods

Animals

Wild type Balb/c mice originating from Charles River Laboratories (Montréal, QC, Canada) were bred in house and used at ages 6–10 weeks. Animal studies were approved by the McGill University Animal Care Committee and performed following the Canadian Council on Animal Care guidelines. Both males and females were used in all experiments. In conducting research using animals, the investigators adhere to the laws of the United States and regulations of the Department of Agriculture.

In vivo antigen and peptide delivery

To investigate DC activation and migration to the MLN, mice were briefly anaesthetized with isoflurane and treated intranasally with 100 µg purified OVA (Worthington Biochemical Corporation, Lakewood NJ) each day for three consecutive days or with a mixture of 100 µg OVA and 50 µg MDP (Peprtech, Montréal QC) (OVA/MDP) on the first day and then OVA alone 24 and 48 h later. Mice were treated with STAT6-IP or STAT6-CP (or saline as a control) twice, 24 and 1 h prior to delivery of OVA/MDP. Mice were sacrificed three hours after the last treatment and lungs collected for flow cytometry or RNA analysis (see below).

Alternatively, mice were sacrificed 48h following the last OVA treatment for collection of MLN for flow cytometry analysis (see below). Other similarly treated mice were allowed to rest for a minimum of 28 days, after which they were briefly anaesthetized as above for intranasal delivery of 100µg OVA (Sigma-Aldrich, Inc., Oakville ON) daily for each of four consecutive days. Lung function was assessed 48 h later, after which mice were

sacrificed to collect lungs, BAL fluid, and blood for further analyses.

Analysis of AHR and airway inflammation

To assess lung function after OVA challenge, mice were anesthetized using xylazine and sodium pentobarbital and paralyzed with pancuronium bromide and total lung resistance and elastance in response to increasing doses of methacholine were measured as previously described [40, 65]. Briefly, anesthetized and paralyzed mice were attached to a computer-controlled small-animal ventilator (flexiVent; Scireq, Montréal, QC, Canada). Baseline respiratory system resistance and maximal resistance to increasing doses of nebulized methacholine were recorded. Afterward, BAL fluid was collected following lavage of the lungs twice with 1 ml PBS. Cells were pooled and the supernatant was collected after centrifugation and stored at -80°C for cytokine-chemokine analysis by multiplex ELISA (Quansys Biosciences, Logan, UT). Total BAL fluid cells were counted before a portion was spun onto microscope slides for staining with Diff-Quick (Fisher Scientific, Ottawa, ON, Canada). Differential cell counts were obtained by counting three to six fields comprising 300 cells per field. Another portion was restimulated *ex vivo* for intracellular cytokine staining (see below). Following BAL fluid collection, blood was collected by cardiac puncture and serum stored at -80°C for analysis of OVA-specific IgE levels.

Lung histology

After BAL fluid collection, lungs were perfused with PBS and inflated with 10% formalin through the trachea. Then, the entire lungs were isolated and placed in formalin. The specimens were embedded in paraffin, cut in $0.5\text{-}\mu\text{m}$ sections, and mounted on glass slides. Slides were stained with haematoxylin and eosin (H&E) or periodic acid-Schiff and examined under a microscope (Olympus BX 51).

Flow cytometry

To characterize lung DC populations, lung tissues were minced and digested for one hour at 37°C in serum-free DMEM (Life Technologies, Carlsbad, CA) containing collagenase XI ($250\mu\text{g/mL}$; Life Technologies); LiberaseTM ($100\mu\text{g/mL}$; Roche, Indianapolis, IN); hyaluronidase 1a (1 mg/mL ; Life Technologies) and DNase I ($200\mu\text{g/mL}$; Sigma-Aldrich) as described previously [65]. The reaction was stopped with EDTA (final concentration 20 mM), and red blood cells were lysed. The remaining cells were washed in PBS twice and resuspended in PBS containing 2% FBS. Then, total cells were counted and extracellular-stained for flow cytometry. All cells for flow cytometry analysis were pre-stained with Fixable Viability Dye eFluor780 (eBioscience) and then treated with anti-mouse CD16/CD32 (Fc block) (BD Biosciences, San Jose CA).

Flow cytometry was done according to the guidelines in Cossarizza et al. [66]

To examine the expression of co-stimulatory molecules on lung and MLN DCs, cells were stained with Pacific Blue-anti-CD45 (BioLegend, San Diego CA), APC-anti-CD11c (BioLegend), PE-Cy7-anti-CD11b (eBioscience, San Diego CA), Alexa Fluor[®] 488-anti-F4/80 (eBioscience), BV510-anti-MHCII (BioLegend), PerCP-anti-CD86 (BioLegend), and PE-anti-OX40L (eBioscience).

To quantify IL-4 production from CD4^{+} T cells by flow cytometry, BAL fluid cells ($10^6/\text{mL}$) were incubated in RPMI 1640 media (Life Technologies) containing 1% penicillin/streptomycin, 10% heat-inactivated feral bovine serum (Life Technologies) with PMA (50 ng/mL) and ionomycin (500 ng/mL) and GolgiStopTM ($0.65\mu\text{L/mL}$) from BD Cytofix/CytopermTM kit (BD Biosciences) for 4 h prior to staining with Pacific Blue-anti-CD4 (BD Biosciences), APC-anti-CD25 (BD Biosciences), and intracellular staining with PE-anti-IL-4 (BioLegend). The same preparation was used to quantify IL-13 production by CD4^{+} T cells from co-culture (see below).

For *in vitro* co-culture experiments, purity of naïve CD4^{+} T cells harvested from spleens of DO11.10 mice (see below) was verified by staining with Pacific-Blue-anti-CD4 (BD Biosciences), FITC-anti-CD62L (eBioscience), and PE-Cy7-anti-CD44 (eBioscience). Following co-culture with naïve CD4^{+} T cells, T cells were stained with Pacific Blue-anti-CD4 (BD Biosciences) and PE-Cy7-anti-IL-13 (eBioscience). The intracellular staining was performed with the BD Cytofix/CytopermTM kit (BD Biosciences). For all flow cytometry analysis, fluorescence minus one (FMO) controls were used to define positive populations. Acquisition was performed on either BD LSRIITM, BD FACSCantoTM (Meakins-Christie Laboratories), or BD LSRFortessaTM (Immunophenotyping Core Facility, RI-MUHC) flow cytometers. Analyses were completed with FlowJoTM software (FlowJo LLC, Ashland OR).

Quansys

The levels of chemokines in BAL fluid were assayed by mouse custom Q-Plex array (Quansys Biosciences, Logan UT). The plates were read with Q-View Imager LS and analyzed with Q-View Software (Quansys Bioscience)

Measurement of OVA-specific IgE

OVA-specific IgE levels were assessed using mouse IgE ELISA set with modifications (BD OptEIATM). Briefly, ELISA plates were coated with $20\mu\text{g/mL}$ OVA in 0.1M bicarbonate buffer (NaHCO_3) and serum samples were incubated with Protein A/G sepharose beads in PBS overnight at 4°C . On the second day, plates were washed with PBS-Tween 20 (0.05%) and blocked with assay diluent after which serum samples were added and incubated overnight at 4°C . The next day, plates were washed with PBS-Tween 20 (0.05%) 5 times, and incubated for 1 h with working detector mix (detection antibody and SAV-HRP

reagent from the same IgE ELISA set). Afterward, the plate was washed 7 times, and the reaction stopped with 1M H₃PO₄, prior to detection.

RNA purification and quantification by real time qPCR

Collected lung tissues from each mouse were homogenized in 1 mL of TRIzol® reagent in micro-centrifuge tubes. Then 0.2 mL chloroform (Life Technologies) was added to each tube and incubated at room temperature for 2–3 min. After centrifugation, the upper aqueous phase was collected into new tubes, 0.5 mL of isopropanol was added, and the tubes were incubated at room temperature for 10 min. Pellets were recovered after centrifugation and washed with 75% ethanol and air-dried. The pellets were suspended in 50 µL of DNase/RNase free water (Life Technologies). RNA was quantified with NanoDrop 1000 (Thermo Scientific) and then reverse-transcribed into cDNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) using Oligo(dT)₁₈. Afterward, OX40L and GAPDH as the housekeeping gene were PCR amplified with StepOnePlus™ Real-Time PCR system (Applied Biosystems) using the following primer pairs: OX40L 5'-GGGAAGGGGTTCACCCCCT-3' & 5'-GAAGAGAGTTGCAGGCAGACA-3' [67] and GAPDH 5'-AAGCTTGGCATTGTGGAAGG-3' & 5'-ATGCAGGGATGATGTTCTGG-3'. The analysis was completed in StepOnePlus™ software. Cycle threshold (Ct) values of OX40L were first normalized to Ct values of GAPDH before quantification by the comparative threshold cycle method to obtain the mRNA levels of OX40L from OVA/MDP- (and peptide-) treated mice relative to the OVA control group.

In vitro BMDC:CD4⁺ T cell co-cultures

BMDCs from wild type Balb/c mice were generated as described previously [68]. Briefly, the tips of both femurs and tibias were carefully cut off and bone marrow cells collected by flushing once from each end with 3 mL of BMDC media (10% FBS, 1% penicillin/streptomycin, 50 µM 2-mercaptoethanol, 1% MEM (minimum essential medium) non-essential amino acids solution, 1% sodium pyruvate in L-glutamine containing RPMI 1640 medium). The isolated cells (0.75 × 10⁶) were plated in 6-well plates in 3 mL of BMDC media containing 20 µg/mL GM-CSF (PeproTech, Montreal, QC) in each well (Day 0). Another 3 mL of fresh BMDC media containing GM-CSF was added to the culture on day 3. On days 6 and 9, 3 mL of the total 6 mL was again replaced with fresh media containing GM-CSF. On day 10, BMDCs were harvested for co-cultures. BMDCs were incubated overnight with saline or with TSLP (100 ng/mL), either alone or with STAT6-IP or STAT6-CP (100 µM). Cells were washed in PBS twice, and then pulsed with OVA peptide (323–339) (100 ng/mL) for 2 h. Naïve CD4⁺ T cells (CD4⁺CD62L^{hi}CD44^{low}) were isolated from spleens of DO11.10 OVA-TCR transgenic mouse by Naïve CD4⁺ T cell selection kit (Miltenyi Biotech, Auburn CA). BMDCs were cultured with the

isolated naïve CD4⁺ T cells in ratio of 1:2 and 48 h later, cells were harvested for flow cytometry analysis as described above.

Statistical analysis

Data were analyzed using Prism 6 (GraphPad software, La Jolla CA) by one-way ANOVA with Newman-Keuls multiple comparison tests. Data are presented as means ± SEMs and *p*-values less than 0.05 were considered statistically significant. Some data (serum IgE levels, in vitro IL-13 producing CD4⁺ T cells) were normalized to OVA/MDP (100%) prior to the statistical tests, as indicated in the figure legends.

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References

- 1 Fahy, J. V., Type 2 inflammation in asthma—present in most, absent in many. *Nat. Rev. Immunol.* 2015. 15: 57–65.
- 2 Lambrecht, B. N. and Hammad, H., The immunology of asthma. *Nat. Immunol.* 2015. 16: 45–56.
- 3 Woodruff, P. G., Modrek, B., Choy, D. F., Jia, G., Abbas, A. R., Ellwanger, A., Koth, L. L. et al., T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am. J. Respir. Crit. Care Med.* 2009. 180: 388–395.
- 4 Hamid, Q. A. and Cameron, L. A., Recruitment of T cells to the lung in response to antigen challenge. *J. Allergy Clin. Immunol.* 2000. 106: S227–S234.
- 5 Carr, T. F., Berdnikovs, S., Simon, H. U., Bochner, B. S. and Rosenwasser, L. J., Eosinophilic bioactivities in severe asthma. *World Allergy Organ J* 2016. 9: 21.
- 6 Humbert, M., The right tools at the right time. *Chest* 2006. 130: 29S–40S.
- 7 Demoly, P., Annunziata, K., Gubba, E. and Adamek, L., Repeated cross-sectional survey of patient-reported asthma control in Europe in the past 5 years. *Eur Respir Rev* 2012. 21: 66–74.
- 8 Nathan, R. A., Thompson, P. J., Price, D., Fabbri, L. M., Salvi, S., Gonzalez-Diaz, S., Maspero, J. F. et al., Taking aim at asthma around the world: global results of the asthma insight and management survey in the Asia-

- Pacific Region, Latin America, Europe, Canada, and the United States. *J Allergy Clin Immunol Pract* 2015. **3**: 734–742 e735.
- 9 Price, D., Fletcher, M. and van der Molen, T., Asthma control and management in 8,000 European patients: the REcognise Asthma and Link to Symptoms and Experience (REALISE) survey. *NPJ Prim Care Respir Med* 2014. **24**: 14009.
 - 10 Bagnasco, D., Ferrando, M., Varricchi, G., Passalacqua, G. and Canonica, G. W., A critical evaluation of anti-IL-13 and anti-IL-4 strategies in severe asthma. *Int Arch Allergy Immunol* 2016. **170**: 122–131.
 - 11 Patel, T. R. and Sur, S., IgE and eosinophils as therapeutic targets in asthma. *Curr. Opin. Allergy Clin. Immunol.* 2017. **17**: 42–49.
 - 12 Wenzel, S., Ford, L., Pearlman, D., Spector, S., Sher, L., Skobieranda, F., Wang, L. et al., Dupilumab in persistent asthma with elevated eosinophil levels. *N. Engl. J. Med.* 2013. **368**: 2455–2466.
 - 13 Besnard, A. G., Togbe, D., Guillou, N., Erard, F., Quesniaux, V. and Ryffel, B., IL-33-activated dendritic cells are critical for allergic airway inflammation. *Eur. J. Immunol.* 2011. **41**: 1675–1686.
 - 14 Mitchell, P. D. and O'Byrne, P. M., Biologics and the lung: TSLP and other epithelial cell-derived cytokines in asthma. *Pharmacol. Ther.* 2017. **169**: 104–112.
 - 15 de Kleer, I. M., Kool, M., de Bruijn, M. J., Willart, M., van Moorleghem, J., Schuijs, M. J., Plantinga, M. et al., Perinatal activation of the interleukin-33 pathway promotes type 2 immunity in the developing lung. *Immunity* 2016. **45**: 1285–1298.
 - 16 Halim, T. Y., Steer, C. A., Matha, L., Gold, M. J., Martinez-Gonzalez, I., McNagny, K. M., McKenzie, A. N. et al., Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 2014. **40**: 425–435.
 - 17 Bell, B. D., Kitajima, M., Larson, R. P., Stoklasek, T. A., Dang, K., Sakamoto, K., Wagner, K. U. et al., The transcription factor STAT5 is critical in dendritic cells for the development of TH2 but not TH1 responses. *Nat. Immunol.* 2013. **14**: 364–371.
 - 18 Han, H., Headley, M. B., Xu, W., Comeau, M. R., Zhou, B. and Ziegler, S. F., Thymic stromal lymphopoietin amplifies the differentiation of alternatively activated macrophages. *J. Immunol.* 2013. **190**: 904–912.
 - 19 Kurowska-Stolarska, M., Stolarski, B., Kewin, P., Murphy, G., Corrigan, C. J., Ying, S., Pitman, N. et al., IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J. Immunol.* 2009. **183**: 6469–6477.
 - 20 Byers, D. E., Alexander-Brett, J., Patel, A. C., Agapov, E., Dang-Vu, G., Jin, X., Wu, K. et al., Long-term IL-33-producing epithelial progenitor cells in chronic obstructive lung disease. *J. Clin. Invest.* 2013. **123**: 3967–3982.
 - 21 Bouffi, C., Rochman, M., Zust, C. B., Stucke, E. M., Kartashov, A., Fulkeron, P. C., Barski, A. et al., IL-33 markedly activates murine eosinophils by an NF-kappaB-dependent mechanism differentially dependent upon an IL-4-driven autoinflammatory loop. *J. Immunol.* 2013. **191**: 4317–4325.
 - 22 Chu, D. K., Llop-Guevara, A., Walker, T. D., Flader, K., Goncharova, S., Boudreau, J. E., Moore, C. L. et al., IL-33, but not thymic stromal lymphopoietin or IL-25, is central to mite and peanut allergic sensitization. *J. Allergy Clin. Immunol.* 2013. **131**: 187–200 e181–e188.
 - 23 Duan, W., Mehta, A. K., Magalhaes, J. G., Ziegler, S. F., Dong, C., Philpott, D. J. and Croft, M., Innate signals from Nod2 block respiratory tolerance and program T(H)2-driven allergic inflammation. *J. Allergy Clin. Immunol.* 2010. **126**: 1284–1293 e1210.
 - 24 Flynn, S., Toellner, K. M., Raykundalia, C., Goodall, M. and Lane, P., CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blr-1. *J. Exp. Med.* 1998. **188**: 297–304.
 - 25 Hammad, H., Chieppa, M., Perros, F., Willart, M. A., Germain, R. N. and Lambrecht, B. N., House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 2009. **15**: 410–416.
 - 26 Ito, T., Wang, Y. H., Duramad, O., Hori, T., Delespesse, G. J., Watanabe, N., Qin, F. X. et al., TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J. Exp. Med.* 2005. **202**: 1213–1223.
 - 27 Magalhaes, J. G., Rubino, S. J., Travassos, L. H., Le Bourhis, L., Duan, W., Sellge, G., Geddes, K. et al., Nucleotide oligomerization domain-containing proteins instruct T cell helper type 2 immunity through stromal activation. *Proc Natl Acad Sci U S A* 2011. **108**: 14896–14901.
 - 28 Seshasayee, D., Lee, W. P., Zhou, M., Shu, J., Suto, E., Zhang, J., Diehl, L. et al., In vivo blockade of OX40 ligand inhibits thymic stromal lymphopoietin driven atopic inflammation. *J. Clin. Invest.* 2007. **117**: 3868–3878.
 - 29 Soumelis, V., Reche, P. A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M. et al., Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol.* 2002. **3**: 673–680.
 - 30 Ito, T., Wang, Y. H., Duramad, O., Hanabuchi, S., Perng, O. A., Gilliet, M., Qin, F. X. et al., OX40 ligand shuts down IL-10-producing regulatory T cells. *Proc Natl Acad Sci U S A* 2006. **103**: 13138–13143.
 - 31 Rogers, P. R., Song, J., Gramaglia, I., Killeen, N. and Croft, M., OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity* 2001. **15**: 445–455.
 - 32 Soroosh, P., Ine, S., Sugamura, K. and Ishii, N., Differential requirements for OX40 signals on generation of effector and central memory CD4+ T cells. *J. Immunol.* 2007. **179**: 5014–5023.
 - 33 Arima, K., Watanabe, N., Hanabuchi, S., Chang, M., Sun, S. C. and Liu, Y. J., Distinct signal codes generate dendritic cell functional plasticity. *Sci Signal* 2010. **3**: ra4.
 - 34 Iwata, A., Kawashima, S., Kobayashi, M., Okubo, A., Kawashima, H., Suto, A., Hirose, K. et al., Th2-type inflammation instructs inflammatory dendritic cells to induce airway hyperreactivity. *Int. Immunol.* 2014. **26**: 103–114.
 - 35 Lutz, M. B., Schnare, M., Menges, M., Rossner, S., Rollinghoff, M., Schuler, G. and Gessner, A., Differential functions of IL-4 receptor types I and II for dendritic cell maturation and IL-12 production and their dependency on GM-CSF. *J. Immunol.* 2002. **169**: 3574–3580.
 - 36 Maroof, A., Penny, M., Kingston, R., Murray, C., Islam, S., Bedford, P. A. and Knight, S. C., Interleukin-4 can induce interleukin-4 production in dendritic cells. *Immunology* 2006. **117**: 271–279.
 - 37 Medoff, B. D., Seung, E., Hong, S., Thomas, S. Y., Sandall, B. P., Duffield, J. S., Superman, D. A. et al., CD11b+ myeloid cells are the key mediators of Th2 cell homing into the airway in allergic inflammation. *J. Immunol.* 2009. **182**: 623–635.
 - 38 McCusker, C. T., Wang, Y., Shan, J., Kinyanjui, M. W., Villeneuve, A., Michael, H. and Fixman, E. D., Inhibition of experimental allergic airways disease by local application of a cell-penetrating dominant-negative STAT-6 peptide. *J. Immunol.* 2007. **179**: 2556–2564.
 - 39 Srinivasa, B. T., Fixman, E. D. and Ward, B. J., Inhibition of STAT6 during vaccination with formalin-inactivated RSV prevents induction of Th2-cell-biased airway disease. *Eur. J. Immunol.* 2014. **44**: 2349–2359.
 - 40 Srinivasa, B. T., Restori, K. H., Shan, J., Cyr, L., Xing, L., Lee, S., Ward, B. J. et al., STAT6 inhibitory peptide given during RSV infection of neonatal mice reduces exacerbated airway responses upon adult reinfection. *J. Leukoc. Biol.* 2017. **101**: 519–529.
 - 41 Wang, Y., Li, Y., Shan, J., Fixman, E. and McCusker, C., Effective treatment of experimental ragweed-induced asthma with STAT-6-IP, a topically delivered cell-penetrating peptide. *Clin. Exp. Allergy* 2011. **41**: 1622–1630.

- 42 Chu, D. K., Mohammed-Ali, Z., Jimenez-Saiz, R., Walker, T. D., Goncharova, S., Llop-Guevara, A., Kong, J. et al., T helper cell IL-4 drives intestinal Th2 priming to oral peanut antigen, under the control of OX40L and independent of innate-like lymphocytes. *Mucosal Immunol* 2014. 7: 1395–1404.
- 43 Duan, W., So, T. and Croft, M., Antagonism of airway tolerance by endotoxin/lipopolysaccharide through promoting OX40L and suppressing antigen-specific Foxp3⁺ T regulatory cells. *J. Immunol.* 2008. 181: 8650–8659.
- 44 Han, J., Dakhama, A., Jia, Y., Wang, M., Zeng, W., Takeda, K., Shiraishi, Y. et al., Responsiveness to respiratory syncytial virus in neonates is mediated through thymic stromal lymphopoietin and OX40 ligand. *J. Allergy Clin. Immunol.* 2012. 130: 1175–1186 e1179.
- 45 Croft, M., So, T., Duan, W. and Soroosh, P., The significance of OX40 and OX40L to T-cell biology and immune disease. *Immunol. Rev.* 2009. 229: 173–191.
- 46 Ansel, K. M., Djuretic, I., Tanasa, B. and Rao, A., Regulation of Th2 differentiation and IL4 locus accessibility. *Annu. Rev. Immunol.* 2006. 24: 607–656.
- 47 Walford, H. H. and Doherty, T. A., STAT6 and lung inflammation. *JAK-STAT* 2013. 2: e25301.
- 48 Mathew, A., MacLean, J. A., DeHaan, E., Tager, A. M., Green, F. H. Y. and Luster, A. D., Signal transducer and activator of transcription 6 controls chemokine production and T helper cell type 2 cell trafficking in allergic pulmonary inflammation. *J. Exp. Med.* 2001. 193: 1087–1096.
- 49 Michael, H., Li, Y., Wang, Y., Xue, D., Shan, J., Mazer, B. D. and McCusker, C. T., TGF-beta-mediated airway tolerance to allergens induced by peptide-based immunomodulatory mucosal vaccination. *Mucosal Immunol* 2015. 8: 1248–1261.
- 50 Lei, L., Zhang, Y., Yao, W., Kaplan, M. H. and Zhou, B., Thymic stromal lymphopoietin interferes with airway tolerance by suppressing the generation of antigen-specific regulatory T cells. *J. Immunol.* 2011. 186: 2254–2261.
- 51 Jember, A. G., Zuberi, R., Liu, F. T. and Croft, M., Development of allergic inflammation in a murine model of asthma is dependent on the costimulatory receptor OX40. *J. Exp. Med.* 2001. 193: 387–392.
- 52 Arestides, R. S., He, H., Westlake, R. M., Chen, A. I., Sharpe, A. H., Perkins, D. L. and Finn, P. W., Costimulatory molecule OX40L is critical for both Th1 and Th2 responses in allergic inflammation. *Eur. J. Immunol.* 2002. 32: 2874–2880.
- 53 Hoshino, A., Tanaka, Y., Akiba, H., Asakura, Y., Mita, Y., Sakurai, T., Takaoka, A. et al., Critical role for OX40 ligand in the development of pathogenic Th2 cells in a murine model of asthma. *Eur. J. Immunol.* 2003. 33: 861–869.
- 54 Omori, M. and Ziegler, S., Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. *J. Immunol.* 2007. 178: 1396–1404.
- 55 Stier, M. T., Bloodworth, M. H., Toki, S., Newcomb, D. C., Goleniewska, K., Boyd, K. L., Quitalig, M. et al., Respiratory syncytial virus infection activates IL-13-producing group 2 innate lymphoid cells through thymic stromal lymphopoietin. *J. Allergy Clin. Immunol.* 2016. 138: 814–824 e811.
- 56 Padilla, J., Daley, E., Chow, A., Robinson, K., Parthasarathi, K., McKenzie, A. N., Tschernig, T. et al., IL-13 regulates the immune response to inhaled antigens. *J. Immunol.* 2005. 174: 8097–8105.
- 57 Kobayashi, T., Iijima, K., Dent, A. L. and Kita, H., Follicular helper T cells mediate IgE antibody response to airborne allergens. *J. Allergy Clin. Immunol.* 2017. 139: 300–313 e307.
- 58 Noble, A. and Zhao, J., Follicular helper T cells are responsible for IgE responses to Der p 1 following house dust mite sensitization in mice. *Clin. Exp. Allergy* 2016. 46: 1075–1082.
- 59 Hong, G. U., Lim, J. Y., Kim, N. G., Shin, J. H. and Ro, J. Y., IgE and IgA produced by OX40-OX40L or CD40-CD40L interaction in B cells-mast cells re-activate FcepsilonRI or FcalphaRI on mast cells in mouse allergic asthma. *Eur. J. Pharmacol.* 2015. 754: 199–210.
- 60 Pattarini, L., Trichot, C., Bogiatzi, S., Grandclaude, M., Meller, S., Keuylian, Z., Durand, M. et al., TSLP-activated dendritic cells induce human T follicular helper cell differentiation through OX40-ligand. *J. Exp. Med.* 2017. 214: 1529–1546.
- 61 Tahilian, V., Hutchinson, T. E., Abboud, G., Croft, M. and Salek-Ardakani, S., OX40 cooperates with ICOS to amplify follicular Th cell development and germinal center reactions during infection. *J. Immunol.* 2017. 198: 218–228.
- 62 Lutz, M. B., Strobl, H., Schuler, G. and Romani, N., GM-CSF monocyte-derived cells and langerhans cells as part of the dendritic cell family. *Front Immunol* 2017. 8: 1388.
- 63 Karulf, M., Kelly, A., Weinberg, A. D. and Gold, J. A., OX40 ligand regulates inflammation and mortality in the innate immune response to sepsis. *J. Immunol.* 2010. 185: 4856–4862.
- 64 Stuber, E. and Strober, W., The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. *J. Exp. Med.* 1996. 183: 979–989.
- 65 Nakada, E. M., Shan, J., Kinyanjui, M. W. and Fixman, E. D., Adjuvant-dependent regulation of interleukin-17 expressing gammadelta T cells and inhibition of Th2 responses in allergic airways disease. *Respir. Res.* 2014. 15: 90.
- 66 Cossarizza, A., Chang, H. D., Radbruch, A., Akdis, M., Andra, I., Annunziato, F., Bacher, P. et al., Guidelines for the use of flow cytometry and cell sorting in immunological studies. *Eur. J. Immunol.* 2017. 47: 1584–1797.
- 67 Jenkins, S. J., Perona-Wright, G., Worsley, A. G., Ishii, N. and MacDonald, A. S., Dendritic cell expression of OX40 ligand acts as a costimulatory, not polarizing, signal for optimal Th2 priming and memory induction in vivo. *J. Immunol.* 2007. 179: 3515–3523.
- 68 Krawczyk, C. M., Sun, J. and Pearce, E. J., Th2 differentiation is unaffected by Jagged2 expression on dendritic cells. *J. Immunol.* 2008. 180: 7931–7937.

Abbreviations: BAL: bronchoalveolar lavage · DC: dendritic cell · AHR: airway hyperresponsiveness · MDP: muramyl dipeptide · Nod2: nucleotide-binding oligomerization domain-containing protein 2 · OVA: ovalbumin · STAT6: signal transducer and activator of transcription 6 · STAT6-IP or IP: STAT6-inhibitory peptide · STAT6-CP or CP: STAT6-control peptide · MHC class II (MHCII): major histocompatibility complex-class II · TSLP: thymic stromal lymphopoietin · Bcl2: B-cell lymphoma 2 · ILC2: group 2 innate lymphoid cells · RSV: respiratory syncytial virus · MLN: mediastinal lymph nodes · FMO: fluorescence minus one

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STAT6 inhibitory peptide reduces dendritic cell migration to the lymph nodes to control Th2 adaptive immunity in the mouse lung

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Reviewer: 1

Comments to the Author

The study by Lee et al. investigates the use of a STAT6-inhibitory peptide (IP) in a Nod2 agonist driven mouse model of allergic asthma and aims to dissect the immunology behind observed reductions in airway inflammation induced by STAT6-IP. Research from the same group has previously demonstrated the effectiveness of STAT6-IP in reducing airway inflammatory responses in models of allergy and RSV infection and here they investigate how this peptide affects Th2 priming in response to OVA and the Nod2 agonist MDP.

Overall, I found this study to be interesting and well thought as a concept but unfortunately has experimental and analytical flaws that have resulted in the over-selling of the results. Indeed, the message revolves around the impact of STAT6-IP on dendritic cell (DC) populations that are induced by local immunization with OVA/MDP. However, I found the DC-specific data to be unreliable in its current form and the study needs reworking to be suitable for publication. Furthermore, there is also no satisfactory extension on the work already published by the group on STAT6-IP efficacy and allergic airway disease.

My major comments are as follows:

1. The authors state that STAT6-IP reduces OVA/MDP-induced DC activation, based on data on OX40L and MHCII only (Fig. 1D). However, OVA/MDP produced no increase in OX40L+ or MHCII+ DCs compared to OVA itself, which would suggest that MDP has no contributing effect. Furthermore, CD86 expression (should be MFI here not %) was unaffected and other DC activation markers were not assessed (e.g. CD80, CD40). This lack of change (in OX40L) did not correlate with the induction of OX40L mRNA seen in lung tissue in OVA/MDP treated mice (Fig 1B) which itself is modest compared to that seen with other Nod2 agonists (e.g. Duan et al. J Allergy Clin Immunol 2010; 126:1284-93) in similar allergy models. This would suggest that the effect of OVA/MDP on OX40L is actually DC-independent. Unless the authors can prove otherwise, the stated conclusion is inaccurate, as all presented evidence does not correlate together.

REPLY:

In our original analysis, we quantified expression of OX40L, MHCII, and CD86 within the CD11b+ DC population only. We have now included further analyses of these data, quantifying the frequency of total lung CD11b+ DCs expressing OX40L - as well as MHCII & CD86. With this new analysis we show that there is a significant increase in OX40L-expressing DCs in the lungs of mice treated with OVA/MDP vs. OVA (Fig 1D). Moreover, this new analysis is quite similar to our findings from the mRNA analysis of OX40L presented in Fig 1B. The revised data for MHCII+ and CD86-expressing CD11b+ DCs has been moved to Supplemental Fig 1B, C, and the gating strategy is presented in Supplemental Fig 1A.

2. I felt that the flow gating strategy for identification of DCs (Fig 1C) and analysis on OX40L expression

needs reworking. CD11c hi DCs should be assessed only (rather than CD11c mid to hi). For OX40L, the main population of cells is skewed and misshaped (likely to due to compensation issues between fluorochromes) and the positive gate needs resetting to reflect CD11chi events and true OX40L+ events. There appears to significant number of OX40L+ events (notably CD11c mid) that look like outliers because contour plots have been selected and outliers shown. This is usually when overall cell numbers are low. Here, the quality of the flow plots need improving. Thus, the OX40L data in Fig. 1D is inaccurate and unreliable, as a result.

REPLY:

We have re-checked our analysis to ensure there are no issues with compensation between fluorochromes affecting the OX40L signal and we have now presented the data as CD11b+ vs. OX40L in the revised figure. We would like to emphasize that it is rare to find abundant OX40L-expressing DCs in the lung - independent of the model used. Our data, showing few OX40L-positive cells are in agreement with data from a number of different groups (using varied allergic stimuli) – including Duan et al J Allergy Clin Immunol 2010; 126:1284 [OVA/MDP]; Plantinga et al, Immunity 2013; 38:322 [HDM]; Chu et al J Allergy Clin Immunol 2013; 131:187 [HDM]. We are thus confident that the OX40L signal we have quantified is real and biologically meaningful.

3. In secondary OVA challenge experiments (Fig. 2), OVA/MDP treatment during priming induced higher numbers of BAL-resident total cells (Fig. 2C; max number 60×10^4), This effect was ablated by STAT6-IP. The increase in cells was shown to be, in part, eosinophils (Fig 2D) and lymphocytes (no reference to whether T or B lymphocytes?) but not macrophages or neutrophils (Fig S1B). However, the numbers do not add up as, for example, the number of macrophages in OVA treated mice (Fig S1B) are higher than the total cell number (Fig 2C) in experiments of the same design. Can this be explained? If these “lymphocytes” are not CD4 T cells then surely it weakens the proposed message on Th2 immunity?

REPLY:

In our original manuscript we mistakenly included frequencies (and not numbers) of some of the BAL fluid cells, including macrophages - thus leading to this confusion. The revised figures now represent BAL fluid numbers throughout. We did this quantification by counting cells manually after Diff-Quick staining and thus cannot discriminate between T and B cells. However, we have now included quantification by flow cytometry of CD4+ T cells - in the revised Fig 4. While no significant changes were found in total

CD4⁺ T cells (Fig 4C, left-hand panel) there were clear trends for induction by MDP (and MDP + STAT6-CP) and reductions by STAT6-IP. See also below, our reply to comments 4 and 5 from this reviewer.

4. Additionally in Figures 2E and 2F, there is nice data showing how induction of CCL17 and CCL22 production by OVA/MDP is inhibited by STAT6-IP. However, STAT6-CP reduced CCL17 and CCL22 production. This is unexpected. Can the authors explain why? Also, one would assume that such release of T cell chemoattractants (and subsequent inhibition by STAT6-IP) would affect T cell recruitment into the BAL fluid. However, there is no T cell data shown (only “lymphocyte” data). Can the authors provide T cell data here, specifically CD4⁺ T cells in the BAL?

REPLY:

Throughout our studies with STAT6-IP and STAT6-CP, we have sometimes observed inhibition of STAT6-CP that is intermediate between the saline controls and STAT6-IP. We have commented on this in each of our papers. We have long considered that this could be due to modest inhibition of STAT6 by STAT6-CP, because this peptide has a single amino acid change compared to STAT6-IP. We have recently initiated a line of investigation dedicated to more clearly defining mechanisms by which STAT6-CP sometimes exhibits inhibitory activity and we are assessing the possibility that STAT6-CP might also have activity as a host defense peptide. Please see our reply to Comment 3 (above) for our description of BAL fluid CD4⁺ T cells – now shown in Fig 4C.

5. The authors suggest that STAT6-IP delivery at OVA/MDP priming reduces CD4⁺ T cells specific IL-4 production and Th2 differentiation (Figure 4). I have a number of issues with this figure. Firstly, no flow plots are shown for the CD4⁺IL-4⁺ data (Fig.4B) and instead data is represented as normalized bar charts. It would be ideal to see flow plots (to see the gating strategy) and absolute numbers of these cells. Furthermore, it would be nice to see IL-4 production measured in the BAL by ELISA or equivalent. Secondly, the gating strategy used to identify Th2 cells is limited to just CD4 and the IL-33 receptor ST2. The authors justify that ST2 marks Th2 cells from other CD4 T cells. However, this assumption is flawed as it has been proven that ST2 exists on Th1 cells (Baumann et al. PNAS 2015; 112: 4056-4066) along with other T cell subsets. Therefore, this strategy is unreliable and Th2 cells should be identified with further

phenotypic markers (e.g. CXCR4). Furthermore, the gating strategy does not include CD3 and the ST2+ gate requires setting with data from FMOs or better ST2^{-/-} cells. Different types of flow plots are shown as well and these should be consistent. This needs readdressing. Thirdly, the data on CD4⁺ TCM and TEM cells showed by represented by absolute numbers rather than percentages. As the effect appears modest by % alone, the defining statement that STAT6-IP reduces Th2 differentiation and possibly TEM survival (no data to prove this) is exaggerated and not believable.

REPLY:

In the revised version we have made several changes to Fig 4. We have kept the OVA-specific IgE data (Fig 4A). We have now added flow panels showing gating of CD4⁺ T cells in the BAL fluid and then, from this, 3 representative plots for IL-4 producing CD4⁺ T cells in mice treated with OVA/MDP alone or with STAT6-IP or STAT6-CP (Fig 4B). Quantification of these data is in Fig 4C (without normalizing the data). All of our flow cytometry gates are defined using fluorescence minus one (FMO) controls, which is now stated on page 22, lines 506-507. Unfortunately, due to the mild nature of this model, we have been unable to reliably detect IL-4 (or other cytokines) in the BAL fluid by ELISA.

We also quantified IL-13-expressing CD4⁺ T cells in the BAL fluid. These were very few in number, and thus we have not included these data. However, we have added the following statement to the manuscript, to address this (page 11, lines 224-225): "IL-13-expressing CD4⁺ T cells were very few in number, though each cell expressing IL-13 also expressed IL-4 (data not shown)."

The modest changes to TEM and TCM, with suggested gating changes (including removal of ST2 for reasons the reviewer stated), are now presented in Supplemental Fig 3C.

6. Data is presented on IL-13 production by naïve CD4 T cells in vitro upon stimulation by OVA and TSLP treated BMDCs (Fig S2). However, no IL-13 data is presented from in vivo experiments, which I feel is important here given that Th2 cells produce IL-13. A conclusion is then presented that STAT6-IP could directly target DCs to reduce Th2 differentiation. I find this very overstated without some accompanying in vivo evidence much like that presented earlier for IL-4 in T cells and DC markers. The only way to truly show this is to use the same allergy model in DC-depleted BALB/c mice and to then assess the effect of STAT6-IP in vivo on Th2 responses (i.e. IL-13) and cell differentiation. Whilst this may be beyond the scope of this study, it would certainly answer whether STAT6-IP was acting directly on DC population.

REPLY:

We have been careful to not specifically conclude from the data in this manuscript that STAT6-IP directly targets DCs. This is one of the goals of our ongoing experiments. However, the data in Supplemental Fig 3A&B do provide evidence that STAT6-IP could potentially target DCs directly. We have modified the text describing these data to clarify our findings and conclusions. It now reads (pages 11&12, lines 243-259): “While IL-4 producing T cells were few in number and did not differ between groups (data not shown), the frequency of IL-13-expressing T cells was modestly reduced by STAT6-IP, but not STAT6-CP (Supplemental Fig 3A&B). Though these data do not rule out indirect effects of STAT6-IP on DCs to control T cell cytokine production, they do suggest that STAT6-IP could directly target DCs to reduce Th2 differentiation.” Please see also our reply to comment 5 from this reviewer.

With regard to assessing responses in DC-depleted Balb/c mice, we believe this line of investigation is likely to be problematic, because, in the absence of DCs, there will be little to no induction of Th2 responses and allergic airway inflammation.

7. In Figure 5, the authors provide data from lung draining MLNs, which aims to look at DC migration into the MLN upon OVA/MDP treatment and the impact of STAT6-IP intervention. In this experiment, the gating strategy for identifying DCs is flawed as they gate on MHCII⁺ cells that are CD11c low to high. Also, the zebra flow plots should be changed to show actual events. Finally, the total numbers of certain DC populations are represented as normalized data. Absolute numbers need to be shown as a normalized difference can look bigger when cell number is low. Also, why was no statistical significance achieved in Fig 5D? It would also be good to know if Th2 cells migrated into the MLN upon OVA/MDP treatment and the effect of STAT6-IP on this recruitment?

REPLY:

We have modified Fig 5A to include representative gating from a new experiment. Our gating strategy for DCs in the MLN focuses specifically on MHCII^{hi} DCs rather than CD11c^{hi} DCs. We have found (in other experiments in my laboratory) that MLN resident DCs express higher levels of CD11c and lower levels of MHCII. Our gating strategy is in agreement with others (i.e. Gerner et al. *Immunity* 2012; 37:364) and is discussed in a review article by Liu and Nussenzweig, *Immunol Rev* 2010. 234:45. While we have tried to detect OX40L-expressing DCs in the MLN, these cells are remarkably few in number, making

quantification difficult. Thus, we now show data for MHCIIhi and CD86-expressing DCs in the MLN only. We have addressed this limitation in the results section (page 12, lines 271-274): “The frequency of OX40L expressing DCs in the MLN was very low; these cells were subtly increased in OVA/MDP- and OVA/MDP plus STAT6-CP-exposed mice and decreased in mice treated with STAT6-IP, though these changes did not reach statistical significance (data not shown).” We have also added one sentence to the discussion (page 15, lines 349-350) “Unfortunately, it was not possible to reliably quantify OX40L-expressing DCs due to the small number of these cells in the MLN.”

8. Given that the paper proposes a link between DC activation (through OX40L) and Th2 cell priming, it is important to know how OX40 expression is affected on Th2 cells in this allergy model. Can the authors provide data on this?

REPLY:

We, too, are interested in regulation of OX40 on CD4+ T cells in this (and other models) in my laboratory and we have some evidence that OX40 is, indeed, upregulated on CD4+ T cells in the MLN of OVA/MDP-treated mice. However, these data are preliminary and the experiments still ongoing in my laboratory and are thus not included in the current manuscript.

9. Some of the key data in this paper appears to not really extend on data already published by the group using OVA as a local antigen (e.g. McCusker et al. J. Immunol 2007; 179:2556-2564). This published study demonstrates that STAT6-IP reduces OVA-induced BAL fluid inflammation and mucus production. Whilst this study adds MDP to the antigenic stimuli, similar results by STAT6-IP on immune cell recruitment and mucus production are seen in this study. Therefore, I feel this study repeats some elements of that work and thus lacks a degree of novelty.

REPLY:

While some of the experimental outcomes are similar to those in our first STAT6-IP paper, we emphasize that in our other publications investigating allergic airway disease (McCusker et al. J Immunol. 2007 and Wang et al. Clin Exp Allergy 2011), STAT6-IP was delivered at the time of OVA (or ragweed) challenge. The novelty in our current work is that we now show that STAT6-IP, when given at the time of antigen priming, effectively inhibits the induction of type-2 responses in the lung. The differences in these two models are described in the discussion, on pages 13&14, lines 297-323 as follows: “In our original STAT6-IP studies [38, 41], STAT6-IP delivered to sensitized mice at the time of OVA or ragweed challenge,

reduced a number of allergic inflammatory outcomes in the lung... To more precisely examine effects of STAT6-IP in DCs in the lung, we developed a new model, related to a lung tolerance model originally described by Duan, et al., [23] where mice are treated with a purified preparation of OVA in the presence of a Nod2 agonist, MDP... Our model is also related to that of Lei et al., who showed that lung delivery of OVA with recombinant TSLP (bypassing induction by MDP) effectively promotes allergic airways disease upon subsequent OVA challenge [50]. Our data show that STAT6-IP effectively reduced OX40L upregulation at the mRNA level in the lung, and reduced OX40L (and possibly MHCII) expressing lung CD11b+ DCs. STAT6-IP also reduced the ability of these DCs to migrate to the lung draining MLN where they would promote Th2 differentiation... Upon OVA challenge 4-5 weeks later, allergic airway inflammatory responses in STAT6-IP treated mice were diminished.”

Reviewer: 2

Comments to the Author

The manuscript by Lee and colleagues states that an inhibitory peptide targeting STAT6 reduces allergic lung inflammation by downregulating dendritic cell activation, DC-migration to the lymph nodes, and DC-mediated Th2 development. The work addresses an interesting issue and is generally in line with previous observations in the field. Although it is known for 20 years that STAT6 is involved in Th2-mediated inflammation (Kaplan MH, et al. *Immunity*. 1996; Akimoto T, et al, *J Exp Med*. 1998), this work provides new insight into mechanisms behind this observation. Experiments are performed on a valid standard and are well described. Yet, information about age and gender of mice used in the experiments is not provided.

Specific comments:

- The authors have already used STAT6-IP in an earlier study to inhibit experimental allergic airways disease (McCusker et al. *Jl* 2007). However, in the latter study a different animal model was used. Could the authors please comment on the different models and specify the advantage of using the OVA/MDP model in this study. Moreover, the OVA/MDP model described by Duan and colleagues (*Innate Signals from Nod2 Block Respiratory Tolerance and Program Th2 Driven Allergic Inflammation Duan, JACI* 2010) involves alum to generate a Th2 response. Did the authors compare their model with the OVA/MDP model used by Duan et al?

REPLY:

For the first comment, please refer to our reply to comment 9 of the first reviewer.

For the 2nd comment, we have described our reasoning for modifying the protocol originally used by Duan et al. This is presented in the Discussion on page 14, lines 304-315: “To more precisely examine effects of STAT6-IP in DCs in the lung, we developed a new model, related to a lung tolerance model originally described by Duan, et al., [23] where mice are treated with a purified preparation of OVA in the presence of a Nod2 agonist, MDP... While their model relied upon subsequent intraperitoneal OVA/alum sensitization to assess the affect of MDP delivery to the lung, we reasoned that OVA/MDP would be sufficient for Th2 priming, the outcome of which could be assessed simply by subsequent OVA challenge. Our model is also related to that of Lei et al., who showed that lung delivery of OVA with recombinant TSLP (bypassing induction by MDP) effectively promotes allergic airways disease upon subsequent OVA challenge [50].”

- The fact that STAT6-IP inhibits OX40L expression induced by OVA/MDP in lungs suggests that OVA and/or MDP would induce OX40L in a STAT6 dependent way. Can the authors comment on this? Which factor might activate STAT6? Does OX40L depend on STAT6? Can one expect a similar role of STAT6 in an MDP-independent model?

REPLY:

We have described this issue in the discussion on Pages 14,15, lines 329-354: “The precise mechanism by which STAT6-IP interfered with DC responses in the lung and MLN in OVA/MDP-treated mice is not clear. TSLP is a potent inducer of OX40L upregulation on DCs [22, 27] but any link between STAT6 activation by TSLP and subsequent OX40L upregulation does not appear to have been addressed. Data from Arima et al. showed that TSLP induced activation of STAT6 in human myeloid DCs in a manner that was independent of the IL-4 receptor [33]; however, we have been unable to replicate these data in murine cells (data not shown). While TSLP may not activate STAT6 directly, it does have the ability to induce production of both IL-4 and IL-13 [22, 54, 55], both of which could then activate STAT6 in DCs. In addition, the ability of STAT6 to promote DC activation is implicated in the work of Halim et al., who showed that IL-13 produced by ILC2s promoted DC migration to the MLN, though these authors did not

present changes in OX40L in this model. A role for IL-13 in upregulation of costimulatory molecules is, however, provided by data from Padilla et al., who showed that administration of recombinant IL-13 to the lung increases MHCII and CD40 on lung CD11c+ cells in a manner that is completely dependent upon expression of the IL-4R α , one subunit of the cognate IL-13 receptor [56]. While changes in MHCIIhi- or CD86-expressing DCs in the lung were quite modest (or absent) in mice treated with OVA/MDP, these DCs (as well as those expressing CD40 [data not shown]) were significantly reduced in the MLN of STAT6-IP-treated mice. Our data thus implicate MDP-induced IL-13 production in these increases in markers of DC activation. The precise molecular mechanism by which IL-13, STAT6, and STAT6-IP regulate DCs expressing OX40L and other co-stimulatory molecules in both the lung and MLN is an ongoing area of our research.”

- In figure 2D the authors show that, compared to OVA only, OVA/MDP induced eosinophil infiltration is completely blocked by STAT6-IP. This is somewhat surprising, since one would not expect that MDP induced eosinophil infiltration is only mediated by STAT6. Since it has been shown earlier, that IL-4 and IL-13 contribute to eosinophil migration by inducing eotaxin (CCL11) in a STAT6-dependent way (Hoeck et al, JI 2011), it would be interesting to analyse IL-4, IL-5 or CCL11 in the BAL as well.

REPLY:

We agree completely with this reviewer - that eosinophil influx into the BAL fluid is likely mediated by a combination of eosinophil chemokines and IL-5. Unfortunately, due to the mild nature of the OVA/MDP model we were unable to reliably detect any cytokines in the BAL fluid. In addition, while CCL17 and CCL22 were detectable in the BAL fluid, CCL11 was not. This has now been added to the results section on Page 9, lines 183-184.

- Figure 4B. Since the authors speculate that the reduced activation of DCs (due to limited OX40L expression and reduced migratory capacities) results in a block of Th2 development, the resulting CD4+ population should be characterized in more detail. Does STAT6-IP treatment also lead to a reduction of IL-5 and IL-13 producing CD4+ T cells. What about GATA3 expression? Is there a general shift in the T cell

population? Because MDP was shown to block tolerance by suppressing the generation of Treg (Duan et al, JACI 2010), and because STAT6-IP seems to have strong effects in the OVA/MDP model, the question arises: does STAT6-IP allow for recovery of the Treg cell population, thus repressing the Th2 response in the OVA/MDP model?

REPLY:

We, too, are interested in these same questions and they are also part of our ongoing studies using OVA/MDP as well as a 2nd model where mice are treated with OVA + IL-33. Our early data suggest that STAT6-IP does not promote IL-10 and/or IFN γ production, either from BAL fluid CD4⁺ T cells (similar to data shown in Fig 4) or from BMDC-T cell co-cultures (similar to data shown in Supplemental Fig 3). As discussed above (comment 8, Reviewer 1) we have preliminary data quantifying OX40 expression on CD4⁺ T cells in our models, and quantifying GATA-3 is obviously of interest as well. However, these data are not yet definitive and comprise ongoing research in my laboratory. In addition, while we have not quantified IL-5 in this model, we did quantify IL-13, as well as IL-4, in the BAL fluid CD4⁺ T cells. Our reply to Reviewer 1, comment 5, is also applicable here: We also quantified IL-13 expressing CD4⁺ T cells in the BAL fluid. These were very few in number, and thus we have not included these data. However, we have added the following statement to the manuscript, to address this (page 11, lines 224-225):

“IL-13-expressing CD4⁺ T cells were very few in number, though each cell expressing IL-13 also expressed IL-4 (data not shown).”

- Figure 4C: please verify citation 45. This paper refers to $\gamma\delta$ T cells.

REPLY:

Based on comments from Reviewer 1 (see Comment 5 above), we have removed this section of the manuscript, which includes this reference.

Second Editorial Decision

23-Aug-2018

Dear Dr. Fixman,

Thank you for submitting your revised manuscript ID eji.201847534.R1 entitled "STAT6 inhibitory peptide

reduces dendritic cell activation and migration to the lymph nodes to control Th2 adaptive immunity in the lung" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referees are included at the bottom of this letter.

Although the referees have recommended publication, some revisions to your manuscript have been requested. Therefore, I invite you to respond to the comments of the referee and revise your manuscript accordingly.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Please show the fluorochrome axis labels and the scaling for all the flow cytometry dot plots. Failure to do this will result in delays in the re-review process.**

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology. We look forward to receiving your revision.

Yours sincerely,
Laura Soto Vazquez

on behalf of
Prof. Kenneth Murphy

Mrs. Laura Soto Vazquez
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Reviewer: 1

Comments to the Author

The revised manuscript by Lee et al. has appeased a large number of my concerns, especially with regard to data presentation and interpretation and its new form is improved from its original version. They have adequately altered the wording of certain sections of the manuscript and other details have been removed (i.e. ST2 data) which improve the manuscript. However, a few minor concerns remain which are detailed below:

1) The reworked data by the authors indicates that there is a significant increase in OX40L-expressing CD11b+ DCs in the lungs of mice treated with OVA/MDP vs. OVA (Fig. 1C). This data is based on % frequency of live cells, which is usually quantified by a flow cytometer. The authors ideally need to show the total cell numbers of OX40L-expressing CD11b+ DCs per lung, which can be easily calculated from organ cell counts. This is important, as changes in frequencies can appear statistically different whilst changes in total cell numbers can sometimes not. Can authors alter the data in Figure 1C to show total cell numbers replaced figure?

2) Also, the title of the paragraph (page 8, line 120) is stated as "STAT6-IP reduces OVA/MDP-induced DC activation in the lungs". This is misleading given that there was no effect of STAT6-IP on CD86+ DCs and that no other activation markers were assessed in the revised manuscript. Can this be changed to reflect the results accurately ie. OX40L-related only?

3) The authors have addressed my comment regarding IL-4 producing CD4+ T cells and flow plots are nice and illustrate the point compellingly. However, I do have a minor concern with the gating strategy as it appears that cells were not identified as CD3+ T cells before being gated as CD4+ T cells. Whilst most CD4+ cells are CD3+, CD4+ CD3- cells do exist (e.g. NK cells - Bernstein et al 2006 J. Immunol) including those capable of producing IL-4 (Brugnoni et al 1997 Leukemia). I think it's important here to determine that the IL-4 producing CD4+ T cells are mainly CD3+ T cells. Can the authors provide evidence that these IL-4 producing CD4+ cells are CD3+ or perhaps demonstrate CD3 identification in their gating strategy from Fig 4?

4) One other point, the authors state (on p11, lines 205-206) that "these data suggest that STAT6-IP delivery at OVA/MDP priming reduces OVA-specific IgE production, Th2 differentiation and possibly, survival of TEM cells". The conclusion regarding the survival of TEM cells is too overreaching and not accurate without any survival data to back it up so should be removed.

5) The authors have not indicated whether Th2 cells were detected in the MLN upon OVA/MDP treatment (Fig. 5) and the effect of STAT6-IP on this. Are the authors able to provide any evidence as reduced Th2

cell migration to the MLN by STAT6-IP would be a nice result?

6) The overall effects of STAT6-IP are more on DC migration rather than activation. I would suggest the title of the manuscript be altered to not mention DC activation

Reviewer: 2

Comments to the Author

The authors have thoroughly addressed all concerns.

Second Revision – authors' response

Reviewer 1: Comments to the Author

The revised manuscript by Lee et al. has appeased a large number of my concerns, especially with regard to data presentation and interpretation and its new form is improved from its original version. They have adequately altered the wording of certain sections of the manuscript and other details have been removed (i.e. ST2 data) which improve the manuscript. However, a few minor concerns remain which are detailed below:

Comment 1: The reworked data by the authors indicates that there is a significant increase in OX40L-expressing

CD11b+ DCs in the lungs of mice treated with OVA/MDP vs. OVA (Fig. 1C). This data is based on % frequency of live cells, which is usually quantified by a flow cytometer. The authors ideally need to show the total cell numbers of OX40L-expressing CD11b+ DCs per lung, which can be easily calculated from organ cell counts. This is important, as changes in frequencies can appear statistically different whilst changes in total cell numbers can sometimes not. Can authors alter the data in Figure 1C to show total cell numbers replaced figure?

REPLY: We agree with this reviewer that this is the most informative way to present this type of data and we always do this in our work now with other similar models. Unfortunately, at the time we were doing the OVA/MDP experiments, we were counting cells only to stain equal numbers for flow cytometry and thus we do not have the total counts. However, we are confident in our conclusion that OX40L-expressing DC increase in the lungs and that they are reduced by STAT6-IP. In our new models, the changes in both the frequency and number of lung DC are quite similar- whether they increase or decrease. In addition, the OVA/MDP model used in the studies in this manuscript was much more 'mild' compared to our newer models, and thus differences in total lung numbers were quite unlikely, making the frequency data quite reliable, especially given the very small numbers of DC

in the lung. In summary, while we agree that converting frequencies to numbers is the best way to present this type of data, we remain confident that the frequency data we have included in our manuscript is a reliable indicator of what was happening with the DCs in the lung.

Comment 2: Also, the title of the paragraph (page 8, line 120) is stated as “STAT6-IP reduces OVA/MDP-induced DC activation in the lungs”. This is misleading given that there was no effect of STAT6-IP on CD86+ DCs and that no other activation markers were assessed in the revised manuscript. Can this be changed to reflect the results accurately ie. OX40L-related only?

REPLY: We have changed the title of the first paragraph as suggested - replacing “activation” with “OX40L-expressing” – on page 8 line 126.

Comment 3: The authors have addressed my comment regarding IL-4 producing CD4+ T cells and flow plots are nice and illustrate the point compellingly. However, I do have a minor concern with the gating strategy as it appears that cells were not identified as CD3+ T cells before being gated as CD4+ T cells. Whilst most CD4+ cells are CD3+, CD4+ CD3- cells do exist (e.g. NK cells - Bernstein et al 2006 J.

Immunol) including those capable of producing IL-4 (Brugnoni et al 1997 Leukemia). I think it's important here to determine that the IL-4 producing CD4+ T cells are mainly CD3+ T cells. Can the authors provide evidence that these IL-4 producing CD4+ cells are CD3+ or perhaps demonstrate CD3 identification in their gating strategy from Fig 4?

REPLY: Unfortunately, we did not include CD3 antibodies in our gating strategy of BALF CD4+ T cells. There is a large body of literature linking OX40L with differentiation of CD4+ Th2 cells. In addition, the majority of studies in the asthma literature emphasize IL-4 production from Th2 differentiation.

While it is certainly possible that the CD4+IL4+ cells could be cell types other than Th2, we are quite confident that the majority of them are, in fact, Th2 cells (express CD3). We do agree that it could be interesting to identify other IL-4 expressing cells in the BALF (such as NK or NK-T cells) as suggested by this reviewer, but feel this is beyond the scope of our current study.

Comment 4: One other point, the authors state (on p11, lines 205-206) that “these data suggest that STAT6-IP delivery at OVA/MDP priming reduces OVA-specific IgE production, Th2 differentiation and possibly, survival of TEM cells”. The conclusion regarding the survival of TEM cells is too overreaching and not accurate without any survival data to back it up so should be removed.

REPLY: We have changed this sentence so that we no longer implicate TEM cells. This is shown now on page 11, line 210.

Comment 5: The authors have not indicated whether Th2 cells were detected in the MLN upon OVA/MDP treatment (Fig. 5) and the effect of STAT6-IP on this. Are the authors able to provide any evidence as reduced Th2 cell migration to the MLN by STAT6-IP would be a nice result?

REPLY: These important studies are ongoing in my laboratory and form the basis for a new manuscript further defining mechanisms by which STAT6-IP regulates MLN T cell responses in both allergy and virus infection models. Thus, they have not been included in this manuscript.

Comment 6: The overall effects of STAT6-IP are more on DC migration rather than activation. I would suggest the title of the manuscript be altered to not mention DC activation

REPLY: As suggested we have changed the title of the manuscript to remove dendritic cell "activation". We have also emphasized that our studies have been done in mice by adding "mouse lung" to the title.

Reviewer 2: Comments to the Author

The authors have thoroughly addressed all concerns.

Third Editorial Decision

29-Oct-2018

Dear Dr. Fixman,

It is a pleasure to provisionally accept your manuscript entitled "STAT6 inhibitory peptide reduces dendritic cell migration to the lymph nodes to control Th2 adaptive immunity in the mouse lung" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: <https://onlinelibrary.wiley.com/toc/15214141/0/ja>). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,

Ivan Shevchenko

on behalf of

Prof. Kenneth Murphy

Dr. Ivan Shevchenko

Editorial Office

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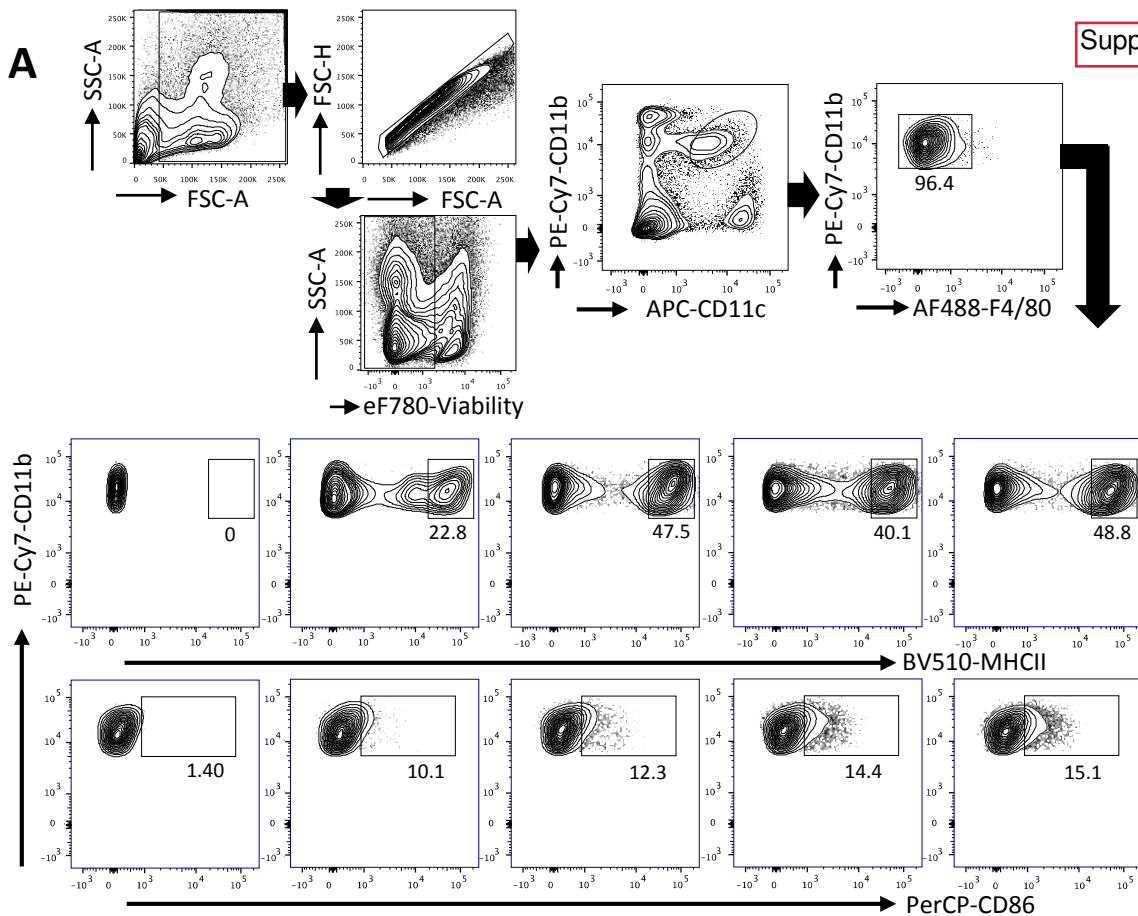
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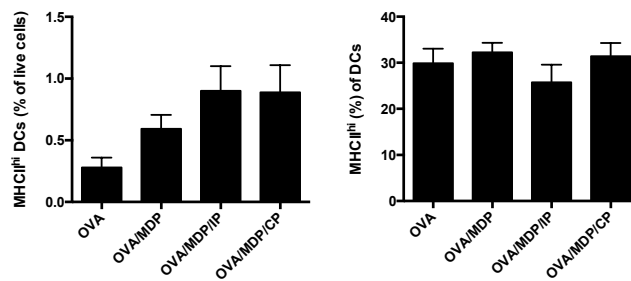
Soojin Lee, Jichuan Shan, Haya Aldossary, Véronique Gaudreault, Mark Bazett
and Elizabeth D. Fixman

**STAT6 inhibitory peptide reduces dendritic cell migration to the lymph nodes to
control Th2 adaptive immunity in the mouse lung**

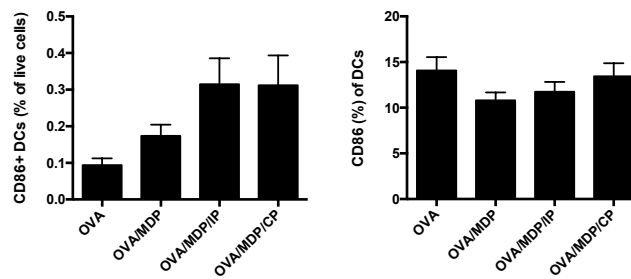
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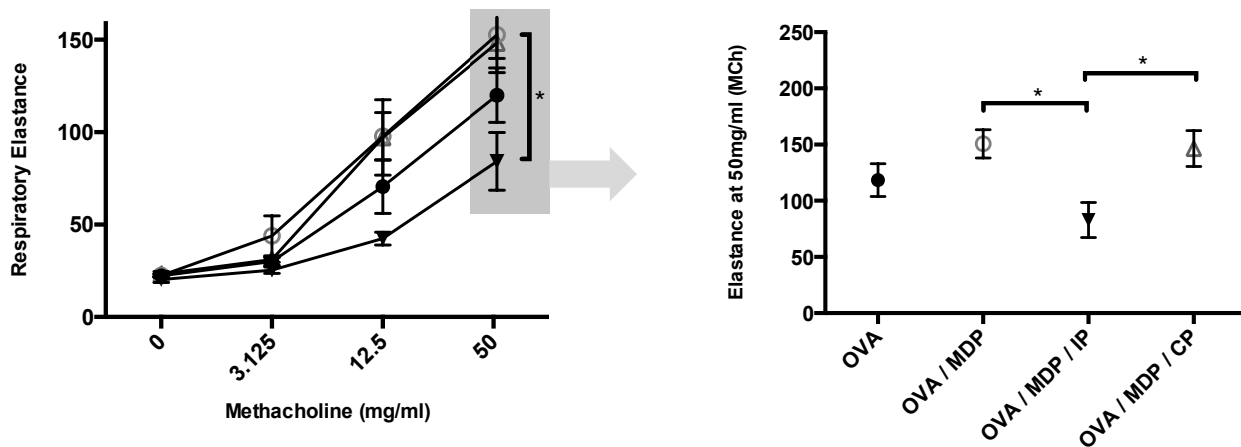
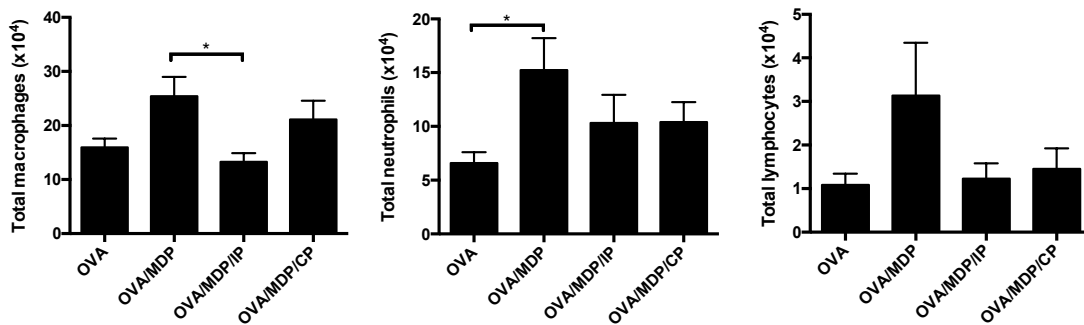
Supporting Figure S1.

A. Mice were treated with OVA, MDP and STAT6-IP or STAT6-CP as described in Fig 1. A. Total lung cells were stained as indicated in *Materials and Methods* and analyzed by flow cytometry. Cells were gated first for viability and singlets (presented on a linear scale), and then as CD11c⁺CD11b⁺F4/80⁻ DCs as shown in Fig 1, followed by gating for expression of MHCIIhi or CD86. Representative flow cytometry plots are presented in B and C and frequencies of CD11b⁺MHCIIhi (B) or CD11b⁺CD86⁺ (C) DCs in the lung (left-hand panels) or frequencies of MHCIIhi or CD86⁺ DCs within the CD11b⁺ DC population (right-hand panels) are shown. Data are presented as the mean +SEM from the combination of 3 independent experiments, with 3 mice per group in each experiment. Outcomes were assessed by one-way ANOVA. No significant differences were found.

A

- △ OVA / MDP / CP
- ▼ OVA / MDP / IP
- OVA / MDP
- OVA

Supp Fig 2

**B**

Supporting Figure S2.

A. Mice were treated with OVA, MDP and STAT6-IP or STAT6-CP as described in Figure 2.

After a minimum of 28 days, mice were challenged with OVA once per day for each of 4 days

and sacrificed 48h later. Lung function was assessed with the flexiVent small animal ventilator

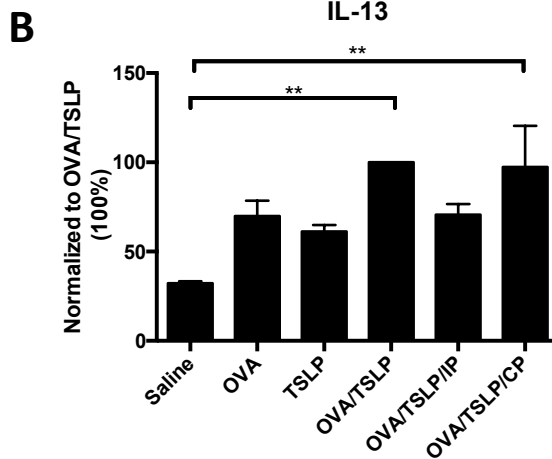
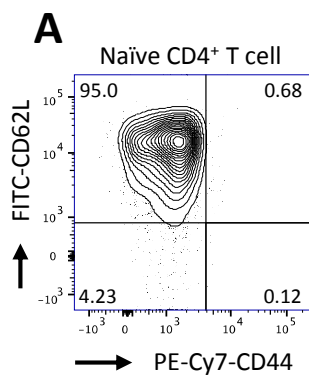
and is presented as total lung elastance: dose response (left) and 50mg/ml dose (right). BAL fluid

was collected for quantification of B. macrophages, C. neutrophils, and D. lymphocytes. **A-E.**

Data are presented as the mean +SEM pooled from 3 independent experiments with 3-4 mice per group in each experiment for A and from 5 independent experiments with 3-5 mice per group in

each experiment for B-D. Outcomes were assessed by one-way ANOVA, followed by Newman-

Keuls multiple comparison test. * $p < 0.05$.



Supporting Figure S3.

A. Phenotype of CD4⁺ T cells purified by magnetic selection: >95% were CD62L⁺CD44⁻ (FITC-CD62L and PE-Cy7-CD44) after selection. B. Following co-culture with BMDCs treated with saline, OVA, TSLP or OVA/TSLP in the presence of STAT6-IP or STAT6-CP, IL-13 expression in the T cells was quantified by flow cytometry as described in the *Materials and Methods*.

Frequencies of IL-13 expressing T cells are presented after first normalizing to the OVA/TSLP group as 100%. Data are presented as the mean +SEM from the combination of 3 independent experiments. Outcomes were assessed by one-way ANOVA, followed by Newman-Keuls multiple comparison test. **p<0.01.