

# Thymic stromal lymphopoietin receptor blockade reduces allergic inflammation in a cynomolgus monkey model of asthma

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**Background:** Thymic stromal lymphopoietin (TSLP) pathway blockade is a potential strategy for asthma treatment because the main activities of TSLP are activation of myeloid dendritic cells (mDCs) and modulation of cytokine production by mast cells. TSLP-activated mDCs prime the differentiation of naive T cells into inflammatory T<sub>H</sub>2 cells.

**Objective:** We sought to investigate mechanisms underlying the development of allergic lung inflammation in cynomolgus monkeys using gene expression profiling and to assess the effect of thymic stromal lymphopoietin receptor (TSLPR) blockade in this model.

**Methods:** An mAb against human TSLPR was generated and confirmed to be cross-reactive to cynomolgus monkey. Animals were dosed weekly with either vehicle or anti-TSLPR mAb for 6 weeks, and their responses to allergen challenge at baseline, week 2, and week 6 were assessed.

**Results:** After 6 weeks of treatment, anti-TSLPR mAb-treated animals showed reduced bronchoalveolar lavage (BAL) fluid eosinophil counts, reduced airway resistance in response to allergen challenge, and reduced IL-13 cytokine levels in BAL fluid compared with values seen in vehicle-treated animals.

Expression profiling of BAL fluid cells collected before and after challenge showed a group of genes upregulated by allergen challenge that strongly overlapped with 11 genes upregulated in dendritic cells (DCs) when *in vitro* stimulated by TSLP (TSLP-DC gene signature). The number of genes differentially expressed in response to challenge was reduced in antibody-treated animals after 6 weeks relative to vehicle-treated animals. Expression of the TSLP-DC gene signature was also significantly reduced in antibody-treated animals.

**Conclusion:** These results demonstrate promising efficacy for TSLPR blockade in an allergic lung inflammation model in

which TSLP activation of mDCs might play a key role. (*J Allergy Clin Immunol* 2013;■■■:■■■-■■■.)

**Key words:** Thymic stromal lymphopoietin, dendritic cells, allergen challenge, cynomolgus monkey, gene expression profiling, eosinophilia, T<sub>H</sub>2 inflammation

Allergic asthma is a common chronic inflammatory disease characterized by eosinophilic inflammation in the lung tissue, increased mucus production, airway hyperresponsiveness (AHR), and structural changes of the airways, including goblet cell hyperplasia and thickening of the submucosa. Even though the cause of the disease is complex and involves both genetic and environmental factors, a common underlying mechanism in patients with allergic asthma is dysregulation of a T<sub>H</sub>2-type inflammatory response.<sup>1</sup>

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine that is thought to play a main role in development of the allergic inflammatory response.<sup>2,3</sup> TSLP is secreted by epithelial cells in response to multiple stimuli, including allergen, viruses, smoke, and cytokines.<sup>4,5</sup> It signals through a heterodimeric receptor formed by the thymic stromal lymphopoietin receptor (TSLPR) and the IL-7 receptor  $\alpha$  chain.<sup>6,7</sup> Myeloid dendritic cells (mDCs) are the main cells that respond to TSLP stimulation, and these TSLP-activated dendritic cells (DCs) possess a unique ability to polarize naive T cells into a T<sub>H</sub>2 phenotype.<sup>8</sup> Other cell types have also been reported to respond to TSLP, including mast cells, basophils, and smooth muscle cells.<sup>5,9-12</sup> In mouse models overexpression of TSLP in the lung and skin resulted in a strong T<sub>H</sub>2-type response.<sup>13,14</sup> Repeated administration of TSLP into the airways of mice results in eosinophilic inflammation that can be blocked by an anti-OX40 ligand antibody, suggesting OX40 ligand upregulation in DCs is an important feature of TSLP-DC activation.<sup>15</sup> In addition, TSLPR knockout mice are resistant to the development of allergic inflammation in the ovalbumin model.<sup>16</sup>

In human subjects TSLP is believed to play a role in allergic diseases, such as atopic dermatitis and asthma. In asthmatic patients TSLP mRNA and protein are upregulated in airway epithelium and airway smooth muscle, and its expression is correlated with lung function.<sup>17,18</sup> Furthermore, polymorphisms in the TSLP gene have been associated with asthma, AHR, IgE levels, and eosinophilia.<sup>19-21</sup>

To investigate the role of TSLP in a primate model of allergic inflammation, we examined the effect of TSLPR blockade using an mAb against TSLPR. The data showed that TSLP plays an important role in the development of allergic responses, as

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**Abbreviations used**

|                    |                                                     |
|--------------------|-----------------------------------------------------|
| AHR:               | Airway hyperresponsiveness                          |
| BAL:               | Bronchoalveolar lavage                              |
| DC:                | Dendritic cell                                      |
| IC <sub>50</sub> : | Inhibitory concentration of 50%                     |
| mDC:               | Myeloid dendritic cell                              |
| TSLP:              | Thymic stromal lymphopoietin                        |
| TSLPR:             | Thymic stromal lymphopoietin receptor               |
| TSLPR mAb:         | Anti-thymic stromal lymphopoietin receptor antibody |

evidenced by its effect on cytokine production and eosinophil infiltration, and TSLPR blockade results in improvement of allergen-induced airway resistance. Expression analysis of bronchoalveolar lavage (BAL) fluid samples suggests that TSLP activates DCs after allergen challenge and that treatment with anti-thymic stromal lymphopoietin receptor antibody (TSLPR mAb) reduces expression of TSLP-response genes in DCs.

**METHODS****Antibody generation**

Armenian hamsters were immunized with soluble TSLPR-Fc fusion protein. Hybridomas were generated from high-titer animals by using P3X63-Ag8653 cells and screened for binding to the antigen by means of ELISA. Subclones were cultured for supernatant generation and purification. Hamster antibodies were ranked according to biochemical and functional properties. A lead candidate was selected, humanized by complementary determining region grafting, fused to an IgG<sub>4</sub> (SPLE) Fc, cloned into expression vectors, and produced in CHO cells.

**DC activation**

DCs and naive T cells were isolated from human blood by using the DC Isolation Kit II and the CD4<sup>+</sup> T Cell Isolation Kit from Miltenyi Biotec (Bergisch Gladbach, Germany). For activation, DCs were incubated with increasing concentrations of TSLPR mAb and then treated with TSLP at 10 ng/mL. After 48 hours, cytokine and surface marker levels were measured.

**Mast cell activation**

Single donor human cord blood-derived CD34<sup>+</sup> cells were purchased from AllCells (Emeryville, Calif). Cells were cultured for 8 weeks in serum-free complete medium (StemPro-34 with supplements) containing human stem cell factor and IL-6. IL-3 was included during the first week. After 8 weeks, cells were stimulated with IL-4 to increase FcεRI levels. Cells were cultured with different concentrations of TSLPR mAb and stimulated with TSLP plus IL-33 (both at 10 ng/mL) overnight. IL-13, IL-5, and GM-CSF levels were measured with kits from Meso Scale Discovery (Birmingham, Ala), according to the manufacturer's instructions.

**Cynomolgus monkey model**

All animal procedures were approved and conducted in compliance with the Charles River Laboratories Institutional Animal Care and Use Committee guidelines (Charles River, Shrewsbury, Mass). Cynomolgus monkeys (*Macaca fascicularis*; 3–10 kg; Charles River) with previously established sensitivity to *Ascaris suum* antigen were used in the study. The study was comprised of 2 separate and consecutive sessions. During session 1, animals were subject to antigen challenge on 2 consecutive days (days 1 and 2) and tested for inflammation and lung function before and after antigen challenge. Animals were divided into 2 groups based on baseline data, receiving either vehicle (n = 8) or TSLP-mAb (7.5 mg/kg administered intravenously, n = 7) weekly for 6 weeks. Allergen challenges were performed at weeks 2 and 6, and inflammation and lung function parameters were assessed. Significance of effects was evaluated by using a nonparametric approach with the Wilcoxon rank sum test.

**Lung function measurements**

A *suum* challenge was performed while each animal was anesthetized by using propofol, in which a single dose of A *suum* antigen was administered through intermittent positive pressure breathing with a ventilator and in-line nebulizer. Acute pulmonary function changes in response to A *suum* challenge were determined during 15 minutes, including the maximum percentage change from baseline that was calculated by using lung resistance and area under the curve during that time period. For assessment of AHR, increasing doses of histamine (beginning with 0.1 μg/kg) were administered intravenously (0.1 ml/kg) 24 hours after the second A *suum* challenge. Lung resistance and dynamic compliance values were continuously measured throughout the histamine dose-response period.

**Expression profiling of BAL samples**

Total RNA was extracted from BAL cell samples by using PerfectPure RNA 96 Cell Kits (catalog no. 2302350; 5 Prime, Hamburg, Germany). Biotinylated and amplified RNA was generated from total RNA by using Affymetrix 3' IVT Express Kits (catalog no. 901229; Affymetrix, Santa Clara, Calif), and samples were subsequently hybridized to Affymetrix HG-U133Plus2.0 microarrays. The microarray data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus under accession number GSE41329.

**Microarray analysis**

The gene expression data were normalized by using the robust multichip average algorithm implemented in Partek Genomics Suite 6.5 (Partek, St Louis, Mo), and ANOVA was used to derive lists of differentially expressed probe sets. Multiple testing correction was performed with the method of Benjamini and Hochberg.<sup>22</sup> For genes with more than 1 probe set, the probe set with the greatest interquartile range across samples was selected to represent the gene. Heatmaps of differentially expressed genes were generated by using unsupervised hierarchic clustering of z score normalized expression values. Differentially expressed genes were identified by requiring fold-change values of at least 1.5-fold and P values of less than .05 corrected for multiple testing. Significance of gene set enrichment was assessed by using the Fisher exact test and gene set enrichment analysis.<sup>23</sup> Activity scores for gene sets were computed by using gene set variation analysis.<sup>24</sup> Analysis was performed with R version 2.13.2 (<http://www.r-project.org>).

**RESULTS****TSLPR mAb characterization**

TSLPR mAb variable regions were derived from an antibody originally generated in hamsters; the antibody was humanized and cloned into an IgG<sub>4</sub> Fc to avoid effector function and subsequently produced in CHO cells. The ability of the TSLPR mAb to bind to the human and cynomolgus monkey receptor was tested by using flow cytometry with transfectant cells expressing human and monkey receptor variants (see the **Methods** section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Concentration-dependent binding curves were obtained in both cases. The calculated dissociation constants were 0.063 ± 0.013 nmol/L for the human receptor and 0.115 ± 0.030 nmol/L for the cynomolgus monkey receptor. In both cases high affinity was observed. The ability of TSLPR mAb to functionally inhibit TSLP-induced responses was tested in a TSLP-induced DC activation assay, in which dose-dependent blockade of CCL17 production induced by TSLP stimulation was demonstrated with an average inhibitory concentration of 50% (IC<sub>50</sub>) of 2.36 ± 0.98 nmol/L for 6 different donors (Fig 1, A). The ability of the antibody to block TSLP-induced signaling in mast cells was also tested in an assay measuring the synergistic effect of TSLP and IL-33 to induce cytokine release in *in vitro*-differentiated human

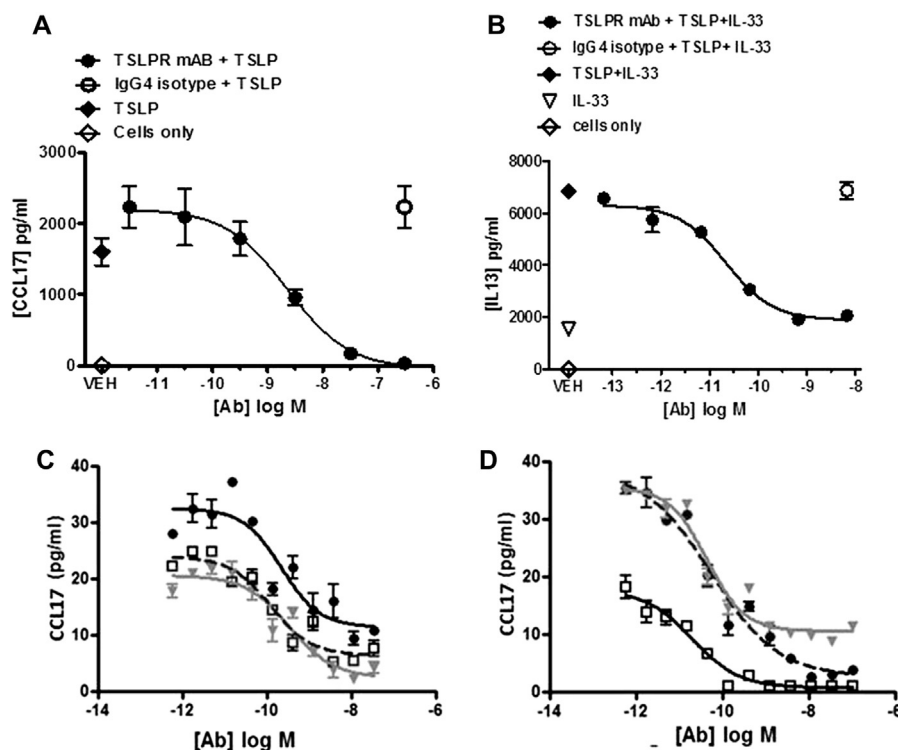


**FIG 1.** A, Inhibition of CCL17 production in DCs by using TSLPR mAb. CCL17 determinations were done 48 hours after stimulation with 10 ng/mL TSLP. Human IgG<sub>4</sub> was used as an isotype control. B, Decrease in IL-13 production by TSLPR mAb after stimulation of *in vitro*-differentiated mast cells with 10 ng/mL each of TSLP and IL-33. C and D, CCL17 production was tested in human (Fig 1, C) and cynomolgus monkey (Fig 1, D) whole blood after stimulation with TSLP in the presence or absence of TSLPR mAb. A representative curve is shown. VEH, Vehicle.

mast cells. In this case a concentration-dependent response was also observed (Fig 1, B), and an average IC<sub>50</sub> of 0.058 ± 0.025 nmol/L was determined for inhibition of IL-13 release in 4 different donors. To assess functional cross-reactivity with cynomolgus monkey, we tested the ability of the antibody to inhibit TSLP-induced CCL17 release in a human and cynomolgus monkey whole-blood assay (Fig 1, C and D). Dose-response curves were generated for 3 donors in each case, where the calculated average IC<sub>50</sub> values were 0.113 ± 0.058 nmol/L for the human assay and 0.039 ± 0.010 nmol/L for the monkey assay.

### Characterization of the *A suum* challenge model in cynomolgus monkeys

Baseline measurements after allergen challenge showed clear signs of allergic inflammation, as previously described in this model.<sup>25,26</sup> Animals exhibited different degrees of allergen-induced bronchoconstriction, AHR to histamine, and pulmonary eosinophilia, as detected in BAL fluid samples collected 8 and 24 hours after challenge. Animals were selected to move on to the dosing phase of the study based on degree of eosinophil infiltrate in BAL fluid and response to histamine at baseline. See Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) for individual data used in the group assignment.

To assess the effect of *A suum* challenge on BAL fluid gene expression, we compared genes expressed in cells from BAL fluid samples collected 8 and 24 hours after challenge against the pre-challenge time point. Five hundred eighty and 662 genes were

differentially expressed at the 8- and 24-hour time points, respectively (see Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Analysis showed that among the significantly enriched pathways were eosinophil-related genes, T<sub>H</sub>2 cytokine response genes, IL-17A, IL-3, IFN-α response genes, and others. For a list of all significantly enriched pathways, see Table E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

Because TSLP mediates the development of allergic inflammation through its effect on mDCs, we hypothesized that TSLP-activated mDC genes might be upregulated by *A suum* challenge in BAL cells. To assess the presence of TSLP-activated mDCs in the model, we performed a separate experiment to identify a TSLP-activated gene signature in mDCs. DCs isolated from human whole blood were stimulated *in vitro* with TSLP and subjected to microarray gene expression profiling. Forty-two genes upregulated by at least 4-fold were chosen to represent a TSLP-activated DC signature (see Fig E1 and Table E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), including known TSLP response genes (CCL17, CD1B, CD86, and CD80), as well as genes not previously described to be regulated by TSLP (ALDH1A2, PPP1R14A, and TM4SF1). We observed 12 and 10 TSLP-activated genes from the *in vitro* mDC experiment that were among the most upregulated genes in BAL fluid samples at the 8- and 24-hour time points, respectively, relative to the unchallenged sample (Fig 2). Fold changes and false discovery rate-corrected *P* values can be found in Table E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). Although eosinophil-specific genes maintained similar levels of induction at both time points

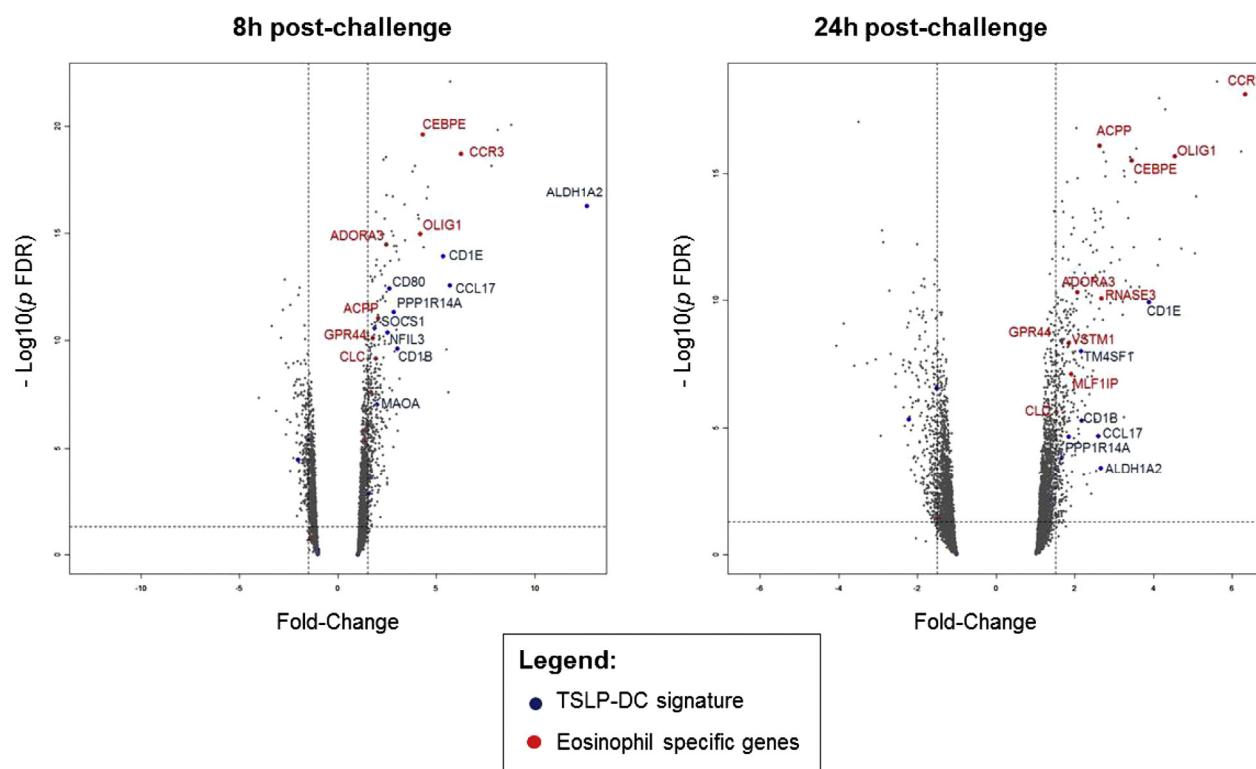


**FIG 2.** Fold change and  $-\log_{10}(P\text{false discovery rate [FDR]})$  values are plotted for all profiled genes 8 and 24 hours after *A suum* challenge at baseline. Red dots indicate genes specifically expressed in eosinophils, whereas blue dots indicate genes in the TSLP-activated DC signature.

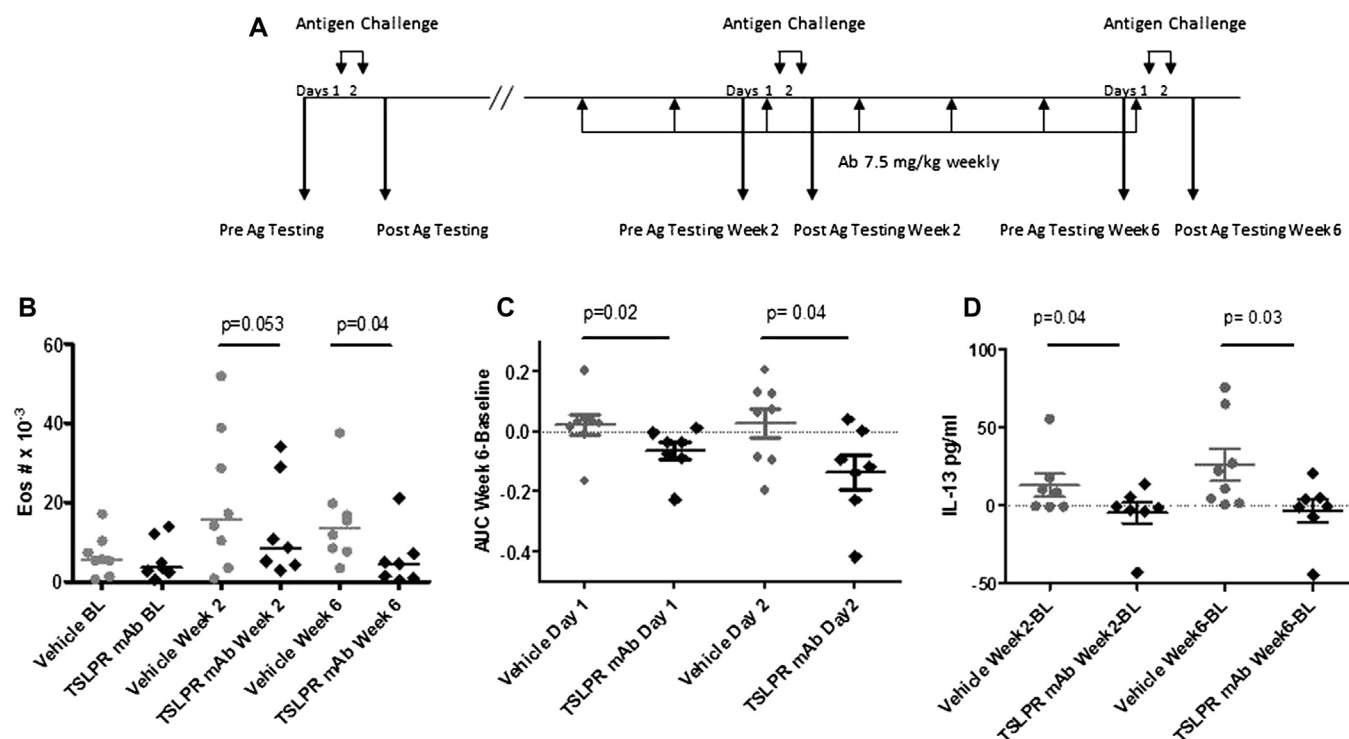


**FIG 3.** **A**, Schematic of the cynomolgus monkey study design. **B**, BAL fluid eosinophil counts after *A suum* challenge expressed as absolute numbers. **C**, IL-13 cytokine levels in BAL fluid (in picograms per milliliter) 8 hours after challenge at week 6 minus baseline levels. **D**, Lung resistance after allergen challenge is shown as the difference in area under the curve (AUC) of week 6 measurements minus baseline measurements for each monkey. Day 1 is after the first challenge, and day 2 is after the second challenge. BL, Baseline.

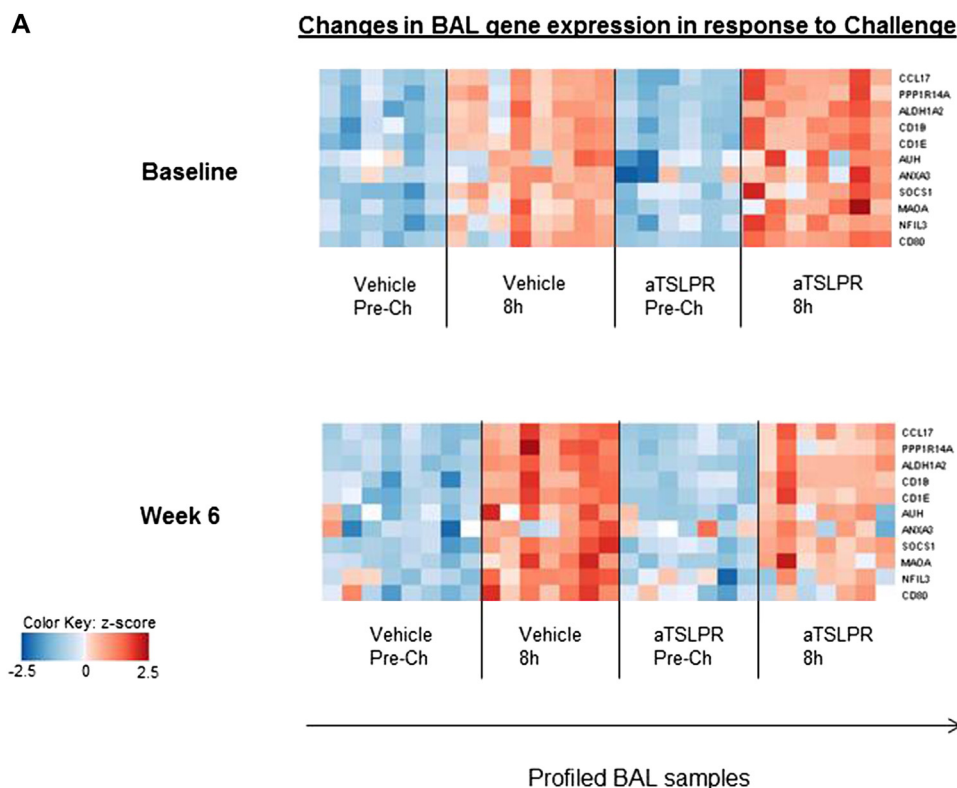


**FIG 4. A**, Expression profiles of 11 coexpressed TSLP-activated DC signature genes displayed as heat maps. *Pre-Ch*, Pre-challenge. **B**, Pathway score indicates the gene set variation analysis score of CCL17, PPP1R14A, ALDH1A2, CD1B, CD1E, AUH, ANXA3, SOCS1, MAOA, NFIL3, and CD80. A positive difference in the pathway score indicates net upregulation of gene set 8 hours after versus before challenge. **C**, Animals were ordered from left to right in terms of increasing pathway scores 8 hours after challenge at week 6. The leftmost animal had the lowest increase in pathway score, whereas the rightmost animal had the greatest increase. *Open bars*, Antibody-treated animals; *gray bars*, vehicle-treated animals. The *y-axis* shows change in response to challenge for (1) IL-13 cytokine levels in BAL fluid (in picograms per milliliter), (2) area under the lung resistance curve (*AUC*), and (3) BAL fluid eosinophil counts (1000 cells/ $\mu$ L).

(consistent with BAL cell count data showing persistent eosinophil influx up to 24 hours), gene set enrichment analysis<sup>23</sup> results showed TSLP-activated DC signature genes were collectively more upregulated at the 8-hour time point relative to the 24-hour time point ( $P = .001$  at 8 hours and  $P = .11$  at 24 hours), suggesting that TSLP-activated DCs might be more prevalent in BAL fluid samples during the early-phase response to allergen. As a control, we tested for the presence of polyinosinic-polycytidylic acid-activated genes<sup>27</sup> in DCs in our study samples, but there was no significant enrichment for these genes at either time point ( $P = .17$  at 8 hours and  $P = .08$  at 24 hours).

### Effects of TSLPR mAb treatment

A subset of animals was selected to proceed for weekly dosing with vehicle ( $n = 8$ ) or TSLPR mAb ( $n = 7$ ) for 6 weeks. Pharmacodynamic measurements showed that CCL17 release in response to TSLP stimulation in whole blood for the vehicle group was consistent for the duration of the study. In contrast, significant decreases for the dosed group were observed throughout the study, indicating the antibody was exerting the expected activity in the blood (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Differential cell counts were obtained at 6 weeks after dosing, and antibody-treated animals showed significantly reduced levels of BAL eosinophil infiltrate compared

with those seen in vehicle-treated animals (Fig 3, B, and see Table E6 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) for full differential counts). Because of the outbred nature of the animals used in the study, some of the measured parameters are variable among subjects. For this reason, most of the data are expressed as the difference between the end points measured after treatment versus the baseline measurements for each animal. IL-13 levels in BAL fluid were increased in vehicle-treated animals in response to challenge at week 6 compared with baseline levels, whereas they were unchanged in TSLPR mAb-treated animals (Fig 3, C). Absolute numbers for IL-13 determinations are shown in Fig E3, E, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). No increases in IFN- $\gamma$  levels were detected during the study (data not shown). In addition to the decrease in inflammatory mediators, a significant reduction in allergen-induced airway resistance was also observed in antibody-treated animals. After adjustment for baseline differences, animals in the TSLPR mAb-treated group exhibited a significant decrease in area under the curve determination for lung resistance compared with animals in the vehicle-treated group (Fig 3, D). This was observed after the first (day 1) and second (day 2) days of allergen challenge. In both cases the reduction was statistically significant. Absolute numbers for these determinations (not corrected by baseline measurements) are shown in Fig E3, A and B. In addition to allergen-induced increases in lung resistance, AHR was tested

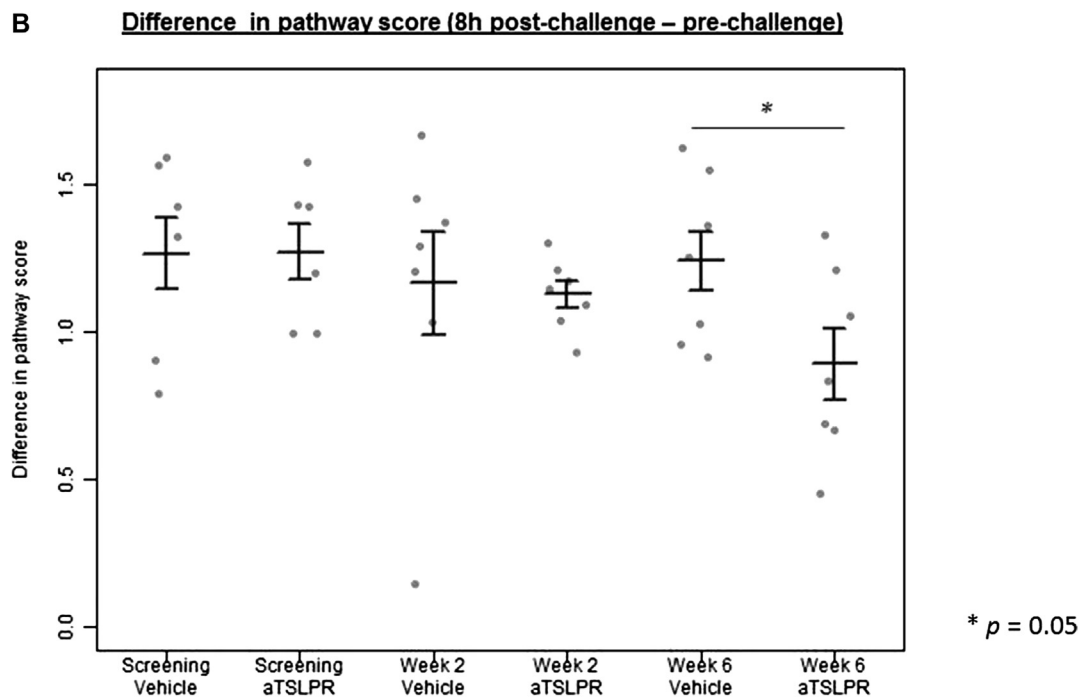


FIG 4. (Continued)

by using different concentrations of histamine. No statistically significant effect of TSLPR mAb was seen. Moreover, data are difficult to interpret because the baseline level for the antibody-treated group was apparently higher in the treated group at baseline compared with the vehicle group (not statistically significant). Absolute and week 6 data compared with baseline values for 3  $\mu\text{g}/\text{kg}$  histamine are shown in Fig E3, C and D, respectively. The differences observed were not significant; however, there was a decreasing trend observed in the antibody-treated group.

In terms of BAL gene expression, although the number of differentially expressed genes in response to challenge remained similar in vehicle-treated animals, the number of differentially expressed genes in antibody-treated animals steadily decreased over 6 weeks of treatment (see Fig E4, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). A similar trend was observed from pathway analysis: after 6 weeks of treatment, antibody-treated animals upregulated fewer relevant pathways in response to challenge compared with baseline (only 1 pathway remained upregulated in the treated group), whereas vehicle-treated animals maintained upregulation of the same baseline pathways (see Table E3).

Not all genes upregulated in the *in vitro* TSLP activation experiment with human donor–derived DCs were detectable in the sampled cynomolgus BAL fluid cells. By filtering for coexpression (spearman correlation > 0.4), we refined the previous 42-gene set to a coexpressed subset of 11 signature genes (CCL17, PPP1R14A, ALDH1A2, CD1B, CD1E, AUH, ANXA3, SOCS1, MAOA, NFIL3, and CD80). These genes were upregulated after *A suum* challenge in vehicle-treated animals but reduced in fold induction after TSLPR mAb treatment (Fig 4, A). A composite pathway score summarizing the global expression pattern of this gene set was derived by using gene set variation analysis.<sup>24</sup> The composite pathway score was increased in response to *A suum* challenge but was significantly less in antibody-versus vehicle-treated animals at the week 6 time point ( $P = .05$ ;

Fig 4, B). We also observed a trend in which animals displaying the greatest reduction in symptoms of allergic inflammation overlapped with animals showing a reduction in pathway activity caused by TSLPR blockade. Ranking animals by the extent of TSLP-DC signature knockdown, we observed that animals with the lowest induction of the TSLP-DC signature in response to *A suum* challenge corresponded to animals with lower levels of IL-13 detected in BAL fluid ( $P = .04$ ; Fig 4, C). Similarly, animals with lower TSLP-DC signature induction tended to have smaller changes in lung resistance and eosinophil influx; however, these trends were not significant ( $P = .09$  and  $P = .24$ , respectively).

## DISCUSSION

In this study we assessed the effect of TSLPR blockade on the inflammatory response to *A suum* challenge in cynomolgus monkeys. The reduction in inflammation and partial improvement in lung function observed in this study confirmed previous findings in other species regarding the importance of the TSLP pathway in the development of allergic inflammation *in vivo*.<sup>16</sup> No increases in  $T_H1$  responses, as measured based on IFN- $\gamma$  production, were observed throughout the study, suggesting that in this model decreases in  $T_H2$  responses do not translate to an increase in  $T_H1$  markers.

Genes induced by challenge in BAL cells were strongly enriched for genes identified *in vitro* to be induced in TSLP-activated DCs, suggesting that allergic inflammation in response to *A suum* challenge in this cynomolgus model likely involves TSLP activation of DCs. In contrast, lack of significant enrichment for polyinosinic-polycytidylic acid-activated genes supports the selective nature of DC activation in this model. By using a previously *in vitro*-identified TSLP-activated DC signature, we were able to detect these rare cells in BAL fluid samples. Detection of DC numbers by using technologies such as flow cytometry has been challenging, given their limited numbers, low abundance, and

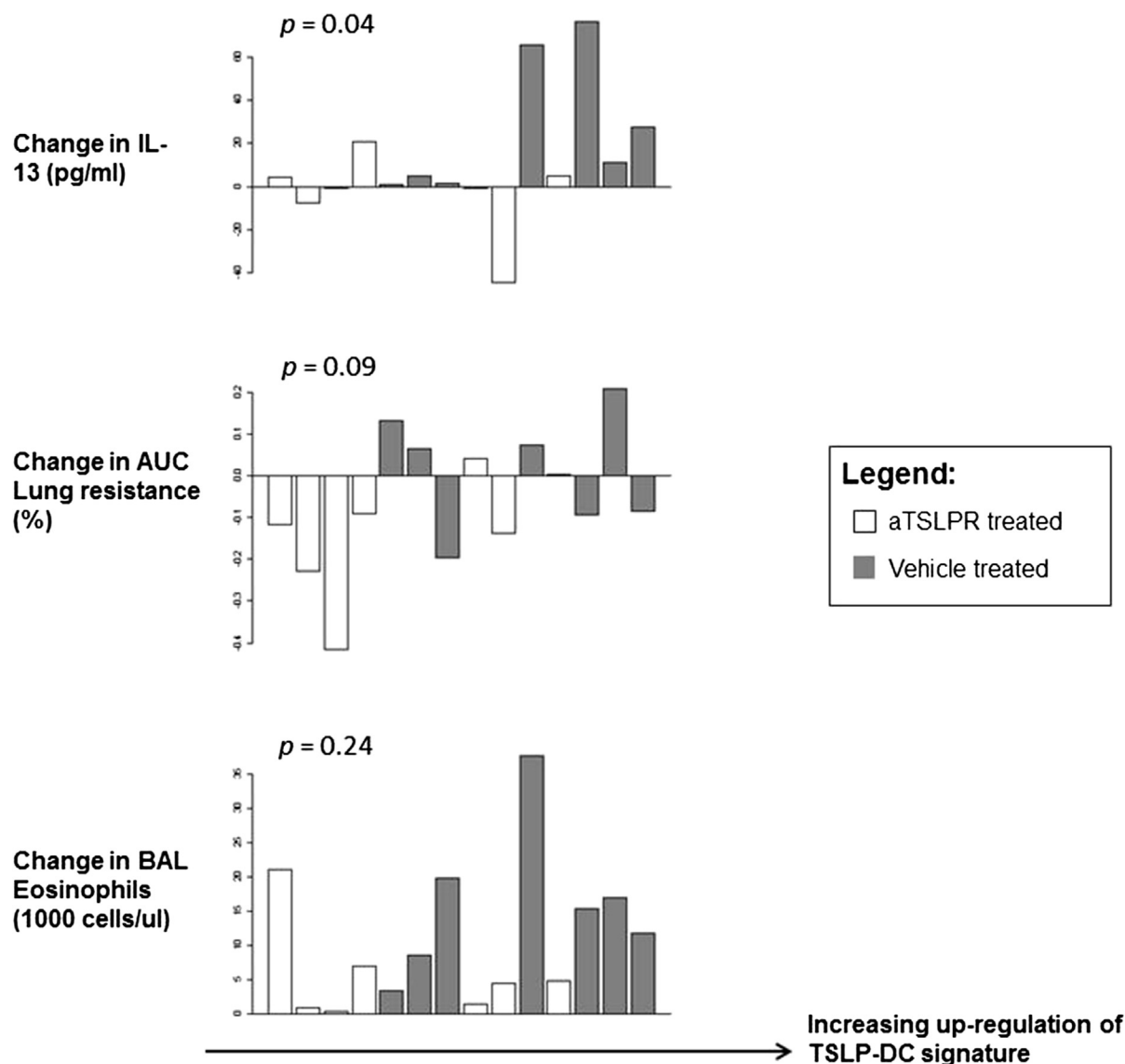


FIG 4. (Continued)

variability of markers depending on subtype and species. Coordinated upregulation of specific DC marker genes is unlikely to occur by chance and thus could suggest increased numbers of TSLP-activated DCs in BAL fluid, change in the activation status of these cells, or both. Because DCs display a strong transcriptional response to TSLP stimulation and most other cell types present in BAL fluid do not respond to TSLP, it is possible that despite being few in number, the robust DC response to TSLP in an allergen challenge setting could constitute an important contribution to the overall gene expression response. It should be noted that we used human microarrays to profile gene expression of cynomolgus BAL cells in this study. Microarray hybridization could be affected by genomic sequence differences across species, and it might be possible, if a cynomolgus-specific microarray was used, that even stronger enrichment for the TSLP-DC signature could be observed.

Enrichment for the TSLP-DC signature appears to be transient (gene signature expression is stronger at 8 hours relative to the 24-hour postchallenge measurement), which might indicate efflux of lung DCs to the lymph nodes after activation by TSLP.

Another potential explanation could be the dilution of the DC signature because of increased recruitment of other cell types in the BAL fluid 24 hours after challenge.

TSLPR mAb-treated animals not only displayed significant reductions in allergic inflammation relative to vehicle-treated animals but also showed reduced expression of an 11-gene TSLP-DC signature in BAL cells. Animals displaying decreased gene signature expression overlapped significantly with animals displaying reductions in IL-13 cytokine levels. A trend was also observed between the extent of gene signature knockdown and reductions in eosinophil influx and lung resistance. The correlation observed between decreased symptoms of allergic inflammation and extent of signature knockdown provides evidence for the hypothesis that in this system TSLP signaling plays a key role in DC activation and maturation and relays and amplifies the inflammatory response triggered by the allergen. Although the gene signature results suggest DCs as drivers of the inflammatory responses in this model, TSLPR blockade could also have an effect on other cell types, such as mast cells or smooth muscle cells, as described *in vitro*.<sup>11,12</sup>

The TSLP-DC signature we identified might serve as a pharmacodynamic marker panel in clinical programs targeting TSLP. Because this signature was expressed in BAL fluid samples in a cynomolgus model, it would be of great interest to test for its presence in induced sputum samples from asthmatic patients in translational studies. The approach described here obviates the need for quantifying sputum cell counts by measuring the presence of a gene expression signature in induced sputum cells.

In summary, the findings in this study support the hypothesis that TSLP is a key mediator of allergic inflammation in multiple species beyond mouse models and suggest that inhibition of the TSLPR pathway could be beneficial for the treatment of patients with allergic asthma. Reduction in allergic inflammation trends with the extent to which TSLP pathway activity is decreased, as evidenced by knockdown of an 11-gene TSLP-activated DC signature. Expression of this signature in induced sputum samples might serve as a pharmacodynamic biomarker for future clinical trials evaluating therapeutics targeting TSLP-mediated activation of DCs, especially in an allergen challenge setting.

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#### Key messages

- A *suum* challenge induces a TSLP-driven allergic inflammatory response in *Ascaris* species-sensitive cynomolgus monkeys.
- TSLPR blockade alleviates allergic lung inflammation by reducing BAL fluid eosinophilia, improving lung function in response to allergen challenge, and reducing IL-13 levels in BAL fluid.
- Genes upregulated in TSLP-stimulated human DCs *in vitro* are also upregulated in BAL cells from cynomolgus monkeys as part of the response to allergen challenge; reduction in TSLP-DC gene expression signature from TSLPR mAb treatment trends with improvement in lung function and reduction of allergic inflammation.

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## **Online Repository Methods**

### **Binding Assay by Flow Cytometry**

The binding affinity of TSLP mAb was determined by applying serial dilutions of the antibody to human and cynomolgus monkey TSLPR expressing cell lines (CHO or HEK293, either transient or stable) or no DNA transfected control CHO cells. The secondary detection reagent was goat F(ab')<sub>2</sub> Anti-Human IgG Alexa Fluor 488 Conjugate( Invitrogen, Carlsbad, CA). FACS data was acquired on an LSRII flow cytometer using FACS Diva software (BD Biosciences, San Jose, CA). At least 10,000 events were acquired for each data point. FACS data was analyzed using Flowjo software (TreeStar, Inc., Ashland, OR) and data was plotted using Prism 5 (GraphPad software, San Diego, CA) with a one site binding model to calculate K<sub>d</sub>.

### **Whole blood CCL17 release assay**

Fresh blood from normal human donors or cynomolgus monkeys was diluted 1:1 with RPMI 1640, incubated with different concentrations of TSLPR mAb and stimulated with 10 ng/ml TSLP for 24h at 37°C. CCL17 was quantitated using a matched anti-human antibody pair from Prosci Bioscience (Poway, California) validated in house for cross-reactivity with cynomolgus monkey. Blood samples from the cynomolgus monkey study were treated in the same manner and this assay was utilized as a pharmacodynamic (PD) measure of treatment effect.

### **Pulmonary function measurement:**

*A. suum* challenge was performed while each animal was anesthetized using propofol (3 mg/kg intravenously) where a single dose of *A. suum* antigen was administered via intermittent positive pressure breathing with a ventilator and in-line nebulizer. Acute pulmonary function changes in response to *A. suum* challenge were determined during 15 min, including the maximum percent change from the baseline that was calculated using lung resistance ( $R_L$ ) and dynamic compliance ( $C_{DYN}$ ) values. For assessment of airway hyperresponsiveness (AHR), increasing doses of histamine (beginning with 0.1  $\mu$ g/kg) were administered intravenously (i.v. 0.1 ml/kg) 24 hours after the second *A. suum* challenge.  $R_L$  and  $C_{DYN}$  values were continuously measured throughout the histamine dose response period. If a positive response occurred, histamine was not administered at the next dose level until the animal's pulmonary function parameters stabilized. Histamine was administered at increasing doses until a strong positive response was seen (~100% increase in  $R_L$  and/or ~50% decrease in  $C_{DYN}$ ) or until the highest dose was administered (30  $\mu$ g/kg).

### **BALF analysis:**

BAL was performed by guiding a pediatric fiber optic bronchoscope past the carina to wedge in a major bronchus. An attempt was made to lavage different lung fields at each time point. Three aliquots of sterile saline (20 ml each) were instilled and aspirated. The first wash was set aside for cytokine analysis and was not combined with subsequent washes. The second and third washes were combined into one collection tube. The cell pellets from the different wash samples were combined for total and differential cell counts. BAL cells were also frozen for subsequent RNA extraction and cytokine/chemokine levels in BAL supernatants were quantified using Milliplex Map Non-Human Primate Cytokine/ Chemokine Immunoassay 23 Plex (catalog #: MPXPRCYTO40KPX23, Millipore Corporation, Billerica, MA) according to the protocol provided by the manufacturer. Fluorescence was

measured by Bio-Plex 200 System powered by the Luminex xMap Technology (Bio-Rad, Hercules, CA, USA). Data acquisition and processing were performed using the Bioplex Manager Software v 6.0 (Bio-Rad, Hercules, CA, USA).

#### **Expression profiling of dendritic cells (DCs) stimulated with TSLP**

DCs were isolated and stimulated as described above. After 6 and 24h post-stimulation, total RNA was extracted from samples using PerfectPure RNA Cultured Cell Kits (5-Prime, Cat no. 2302520). Biotinylated, amplified RNA was generated from total RNA using Illumina® TotalPrep™ RNA Amplification Kits (Life Technologies, Cat. No. AMIL1791) and samples were subsequently hybridized to Illumina Human HT-12 v3 Expression BeadChips.

## ONLINE REPOSITORY FIGURE AND TABLE LEGENDS

### ONLINE REPOSITORY FIGURE LEGENDS

#### Figure E 1:

Expression profile of 1046 genes differentially expressed in dendritic cells in response to TSLP stimulation. UNST: unstimulated, TSLP: TSLP 10 ng/ml stimulation.

#### Figure E 2:

Inhibition of CCL17 release in the whole blood assay in response to TSLP, in vehicle and TSLPR mAb-treated groups. Data is expressed as fold change from un-stimulated blood.

#### Figure E 3:

A) and B) Allergen-induced bronchoconstriction measured as AUC of lung resistance. Data represents absolute values of the measurements. Results after the first and second challenge are shown. Day 1: A) and Day 2: B). C) AHR measurements in response to 3 µg/kg histamine. Lung resistance is shown as AUC post-allergen challenge minus determinations pre-allergen challenge. D) Difference in lung resistance at Week 6 – Baseline for individual monkeys after challenge with 3 µg/kg histamine. BL: baseline. E) Absolute data for IL-13 determinations done in BAL fluid 8 h after challenge.

#### Figure E 4:

A) Number of differentially expressed genes 8h and 24h post *A.suum* challenge in all animals at screening (black), in vehicle and antibody treated animals at Week 6 (dark grey). B) Fold-change differences in expression of TSLP-activated DC signature genes in vehicle (dark grey) and antibody treated animals (white), 8h and 24h post-challenge, after 6 weeks of treatment. Genes on x-axis were ordered from left to right by fold-change in the 8h post-challenge comparison, the left-most gene with the greatest fold-change.

## ONLINE REPOSITORY TABLE LEGENDS

### Table E1:

Relative and absolute eosinophil counts and histamine response data for all monkeys at Baseline.

### Table E2:

Fold-change and FDR corrected p-values of genes differentially expressed in BAL fluid samples at 8h and 24h time points post A.suum challenge.

### Table E3:

Gene set enrichment analysis (GSEA) results on genes up-regulated 8h and 24h post-challenge in vehicle and TSLPR mAb-treated monkeys, at baseline and week 6 timepoints.

### Table E4:

Fold-change and FDR corrected p-values of 42 genes up-regulated at least 4 fold in DCs when stimulated *in vitro* by TSLP.

### Table E5:

Fold-change and FDR corrected p-values of genes in the TSLP-DC signature 8h post A.suum challenge vs. pre-challenge, in vehicle and anti-TSLPR mAb treated animals, after 6 weeks of treatment.

### Table E 6:

Differential cell counts (macrophages, eosinophils, neutrophils, lymphocytes and mast cells) measured pre-challenge and 8h, 24h post-challenge at baseline and 6 weeks after dosing with Vehicle or TSLPR mAb.

### Table E 7:

Fold change and FDR corrected p-values for Eosinophil, TSLP\_DC and polyI:C signature genes, 8h and 24h post-challenge at baseline, week 2 and week 6 timepoints, for vehicle and TSLPR mAb-treated groups.

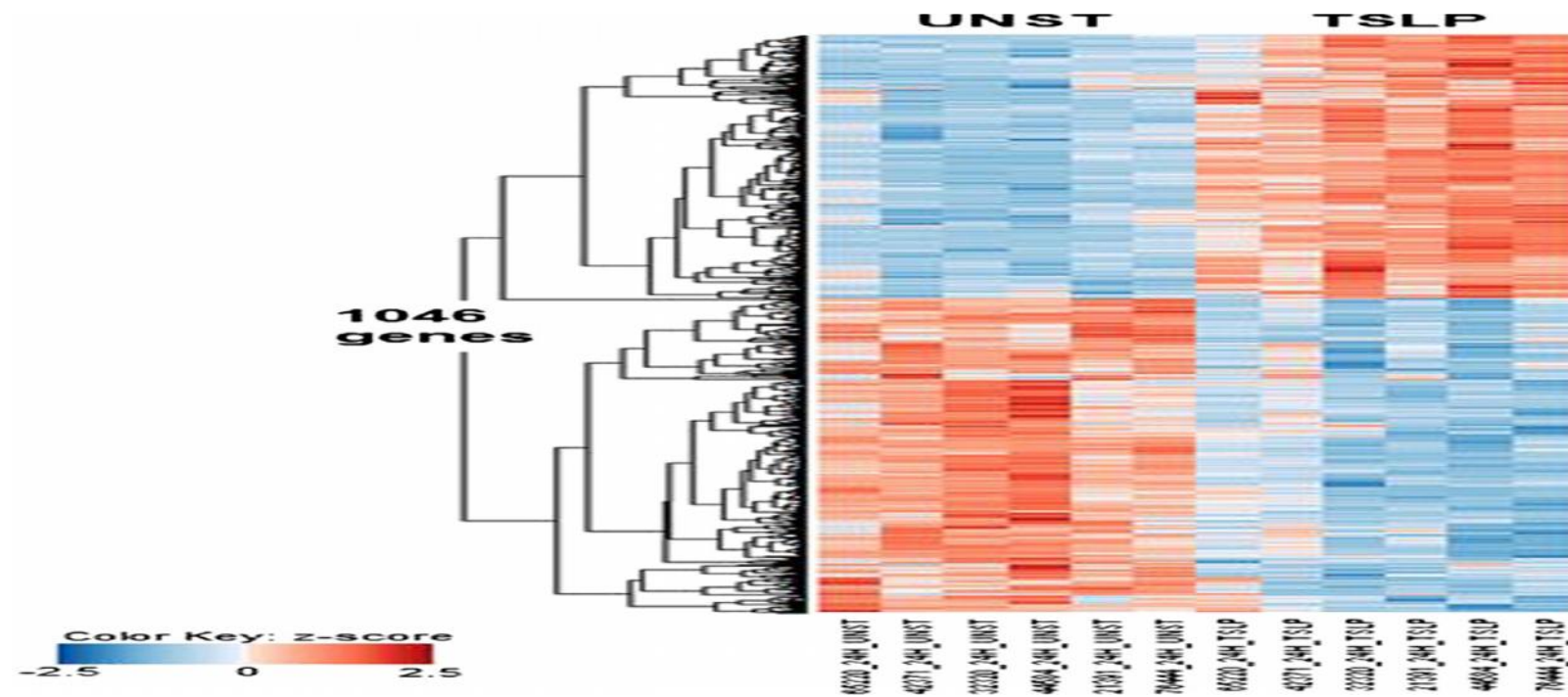


Fig E1

Donavan T. Cheng , Cynthia Ma , Jens Niewoehner , Martin Dahl , Angela Tsai , Jun Zhang , Waldemar Gonsiorek , Su...

**Thymic stromal lymphopoietin receptor blockade reduces allergic inflammation in a cynomolgus monkey model of asthma**

Journal of Allergy and Clinical Immunology null 2013 null

<http://dx.doi.org/10.1016/j.jaci.2013.05.011>

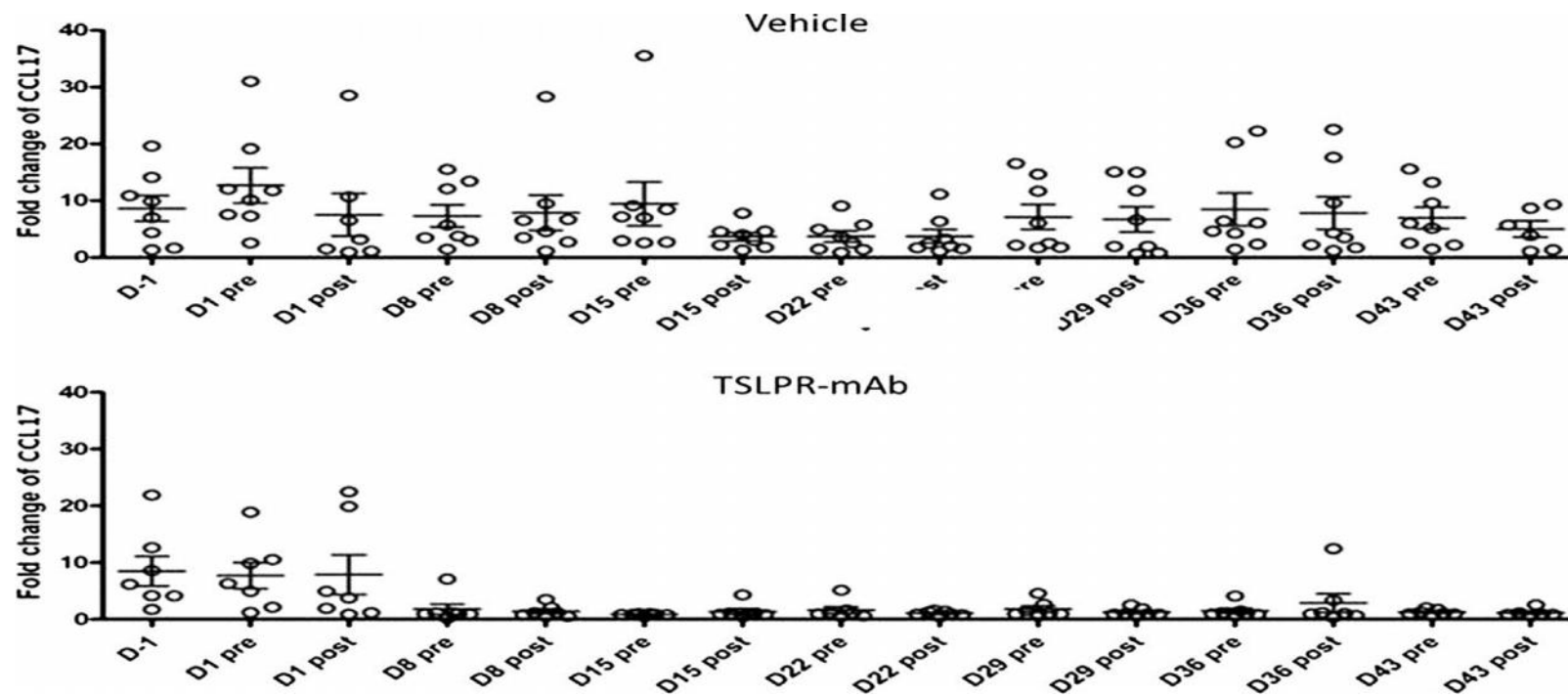


Fig E2

Donavan T. Cheng , Cynthia Ma , Jens Niewoehner , Martin Dahl , Angela Tsai , Jun Zhang , Waldemar Gonsiorek , Su...

**Thymic stromal lymphopoietin receptor blockade reduces allergic inflammation in a cynomolgus monkey model of asthma**

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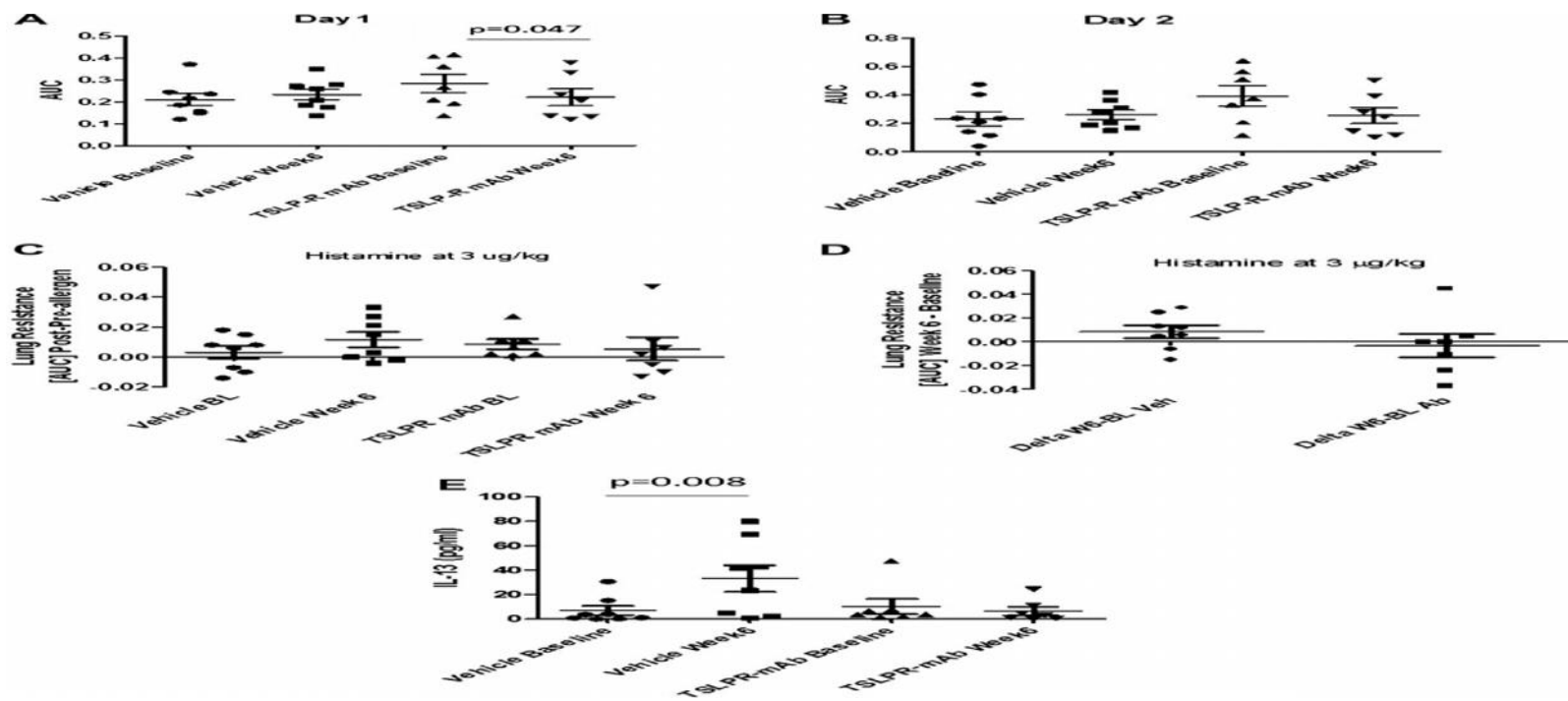


Fig E3

Donavan T. Cheng , Cynthia Ma , Jens Niewoehner , Martin Dahl , Angela Tsai , Jun Zhang , Waldemar Gonsiorek , Su...

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Journal of Allergy and Clinical Immunology null 2013 null

<http://dx.doi.org/10.1016/j.jaci.2013.05.011>



**Table E1: Data for Animal Assignment****Group 1**

| <b>USDA<br/>Number</b>            | <b>Session 1<br/>Study<br/>Number</b> | <b>Session 2<br/>Study<br/>Number</b> | <b>Relative Eosinophils<br/>8 Hours Post Challenge<br/>(%)</b> | <b>Relative Eosinophils<br/>24 Hours Post<br/>Challenge<br/>(%)</b> | <b>Histamine dose<br/>Required to Elicit a<br/>30% Increase in<br/>Resistance after<br/><i>A. suum</i> Challenge<br/>(PC<sub>30</sub>)</b> | <b>Histamine dose<br/>Required to Elicit a<br/>50% Increase in<br/>Resistance after<br/><i>A. suum</i> Challenge<br/>(PC<sub>50</sub>)</b> |
|-----------------------------------|---------------------------------------|---------------------------------------|----------------------------------------------------------------|---------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| 1573                              | 2                                     | 1001                                  | 46                                                             | 50                                                                  | 0.71                                                                                                                                       | 2.04                                                                                                                                       |
| 48-135                            | 3                                     | 1002                                  | 11                                                             | 13                                                                  | 0.74                                                                                                                                       | 1.76                                                                                                                                       |
| 30-83                             | 9                                     | 1101                                  | 60                                                             | 45                                                                  | 2.69                                                                                                                                       | 10.31                                                                                                                                      |
| 124-1156                          | 10                                    | 1003                                  | 36                                                             | 15                                                                  | 1.05                                                                                                                                       | 2.91                                                                                                                                       |
| 54-1187                           | 13                                    | 1004                                  | 43                                                             | 28                                                                  | 0.46                                                                                                                                       | 0.93                                                                                                                                       |
| 105-115                           | 14                                    | 1005                                  | 26                                                             | 77                                                                  | 1.48                                                                                                                                       | 5.93                                                                                                                                       |
| 85-691                            | 18                                    | 1006                                  | 65                                                             | 42                                                                  | 5.41                                                                                                                                       | 44.05                                                                                                                                      |
| 105-152                           | 19                                    | 1007                                  | 77                                                             | 74                                                                  | 1.60                                                                                                                                       | 5.77                                                                                                                                       |
| <b>Mean</b>                       |                                       |                                       | 45                                                             | 43                                                                  | 1.77                                                                                                                                       | 9.21                                                                                                                                       |
| <b>Standard Deviation</b>         |                                       |                                       | 21.6                                                           | 24.2                                                                | 1.6                                                                                                                                        | 14.4                                                                                                                                       |
| <b>Standard Error of the Mean</b> |                                       |                                       | 7.6                                                            | 8.6                                                                 | 0.6                                                                                                                                        | 5.1                                                                                                                                        |

**Group 2**

| <b>USDA<br/>Number</b>            | <b>Session 1<br/>Study<br/>Number</b> | <b>Session 2<br/>Study<br/>Number</b> | <b>Relative Eosinophils<br/>8 Hours Post Challenge<br/>(%)</b> | <b>Relative Eosinophils<br/>24 Hours Post<br/>Challenge<br/>(%)</b> | <b>Histamine dose<br/>Required to Elicit a<br/>30% Increase in<br/>Resistance after<br/><i>A. suum</i> Challenge<br/>(PC<sub>30</sub>)</b> | <b>Histamine dose<br/>Required to Elicit a<br/>50% Increase in<br/>Resistance after<br/><i>A. suum</i> Challenge<br/>(PC<sub>50</sub>)</b> |
|-----------------------------------|---------------------------------------|---------------------------------------|----------------------------------------------------------------|---------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| 54-1182                           | 4                                     | 2001                                  | 72                                                             | 29                                                                  | 1.71                                                                                                                                       | 9.60                                                                                                                                       |
| 113-123                           | 6                                     | 2002                                  | 65                                                             | 50                                                                  | 3.55                                                                                                                                       | 21.09                                                                                                                                      |
| 54-1171                           | 8                                     | 2003                                  | 65                                                             | 78                                                                  | 0.73                                                                                                                                       | 1.83                                                                                                                                       |
| 1531                              | 5                                     | 2004                                  | 50                                                             | 38                                                                  | 1.26                                                                                                                                       | 4.07                                                                                                                                       |
| 124-1150                          | 11                                    | 2005                                  | 39                                                             | 10                                                                  | 0.66                                                                                                                                       | 1.54                                                                                                                                       |
| 124-1320                          | 12                                    | 2006                                  | 37                                                             | 34                                                                  | 0.51                                                                                                                                       | 1.06                                                                                                                                       |
| 54-1153                           | 17                                    | 2007                                  | 56                                                             | 61                                                                  | 1.08                                                                                                                                       | 2.56                                                                                                                                       |
| AD54                              | 20                                    | 2101                                  | 16                                                             | 24                                                                  | 0.48                                                                                                                                       | 1.18                                                                                                                                       |
| <b>Mean</b>                       |                                       |                                       | 50                                                             | 40                                                                  | 1.25                                                                                                                                       | 5.37                                                                                                                                       |
| <b>Standard Deviation</b>         |                                       |                                       | 18.5                                                           | 21.7                                                                | 1.0                                                                                                                                        | 6.9                                                                                                                                        |
| <b>Standard Error of the Mean</b> |                                       |                                       | 6.5                                                            | 7.7                                                                 | 0.4                                                                                                                                        | 2.5                                                                                                                                        |

**Excluded Animals**

| <b>USDA<br/>Number</b> | <b>Session 1<br/>Study<br/>Number</b> | <b>Session 2<br/>Study<br/>Number</b> | <b>Relative Eosinophils<br/>8 Hours Post Challenge<br/>(%)</b> | <b>Relative Eosinophils<br/>24 Hours Post<br/>Challenge<br/>(%)</b> | <b>Histamine dose<br/>Required to Elicit a<br/>30% Increase in<br/>Resistance after<br/><i>A. suum</i> Challenge<br/>(PC<sub>30</sub>)</b> | <b>Histamine dose<br/>Required to Elicit a<br/>50% Increase in<br/>Resistance after<br/><i>A. suum</i> Challenge<br/>(PC<sub>50</sub>)</b> |
|------------------------|---------------------------------------|---------------------------------------|----------------------------------------------------------------|---------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| 54-1161                | 1                                     | NA                                    | 27                                                             | 54                                                                  | 4.91                                                                                                                                       | 49.49                                                                                                                                      |
| 24-96                  | 7                                     | NA                                    | 60                                                             | 48                                                                  | 2.02                                                                                                                                       | 8.85                                                                                                                                       |
| 54-1150                | 15                                    | NA                                    | 64                                                             | 26                                                                  | 1.70                                                                                                                                       | 5.53                                                                                                                                       |
| 125-95                 | 16                                    | NA                                    | 48                                                             | 41                                                                  | 3.26                                                                                                                                       | 20.65                                                                                                                                      |
| 54-1248                | 21                                    | NA                                    | 44                                                             | 79                                                                  | 2.33                                                                                                                                       | 10.82                                                                                                                                      |