Signal Transducer and Activator of Transcription 6 Controls Chemokine Production and T Helper Cell Type 2 Cell Trafficking in Allergic Pulmonary Inflammation

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Abstract

Antigen-specific CD4 T helper type 2 (Th2) cells play a pivotal role in the induction of allergic asthma, but the mechanisms regulating their recruitment into the airways are unknown. Signal transducer and activator of transcription factor (Stat)6 is a transcription factor essential for Th2 cell differentiation. Here we show that Stat6 also controls Th2 cell recruitment and effector function in allergic inflammation in vivo. To isolate the role of Stat6 in regulating Th2 cell trafficking and effector function from its role in Th2 cell differentiation, we used a murine model of asthma in which in vitro–differentiated $Stat6^{+/+}$ antigen-specific Th2 cells were adoptively transferred into naive $Stat6^{-/-}$ and $Stat6^{+/+}$ mice followed by aerosol antigen challenge. We found that all of the features of asthma, including Th2 cell accumulation, Th2 and eosinophil-active chemokine production, and airway eosinophilia, mucus production, and hyperresponsiveness seen in $Stat6^{+/+}$ mice, were dramatically absent in $Stat6^{-/-}$ mice that received $Stat6^{+/+}$ antigen-specific Th2 cells. Our findings establish Stat6 as essential for Th2 cell trafficking and effector function and suggest that interruption of Stat6 signaling in resident cells of the lung is a novel approach to asthma therapy.

Key words: asthma • cytokines • eosinophil • transcription factor • knockout mouse

Introduction

Allergic asthma is a disease of airway inflammation associated with airway hyperresponsiveness (AHR),[1] eosinophilia, and increased mucus production in the lungs (1). $CD4^+$ Th2 cells are believed to play a critical role in controlling the inflammation seen in asthma, as elevated levels of IL-4 and IL-5 have been detected in bronchial biopsies, bronchoalveolar lavage (BAL), and blood of allergic patients compared with normal individuals (2–4).

Murine models of pulmonary inflammation and AHR have elegantly demonstrated that Th2 cells and the cytokines they secrete are essential for the development of eosinophilia and AHR (5–8), validating these observations in humans with allergic asthma. Adoptive transfer of effector Th2 cells into naive mice followed by exposure to inhaled antigen induces the pathophysiological features of asthma, including eosinophilic inflammation, mucus hypersecretion, and AHR (9–11), demonstrating that these cells are fully capable of producing the asthma phenotype. Although it is firmly established that Th2 cells are central to the pathogenesis of asthma, the molecular mechanisms controlling Th2 cell trafficking into the lung are unknown.

Signal transducer and activator of transcription factor (Stat)6 is essential for the induction of IL-4– and IL-13–mediated responses (12–14). Ligation of the IL-4R or IL-13R results in the activation of Stat6 through the phosphorylation by Janus kinase (JAK1 and JAK3; reference 15). In vivo, T cells from Stat6-deficient ($Stat6^{-/-}$) mice are unable to differentiate into Th2 cells in response to IL-4 or IL-13, and B cells from these mice cannot undergo class switching to IgE, suggesting that the Stat6 pathway is important for the allergic response (13, 14). In fact, pulmo-

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[1]*Abbreviations used in this paper:* AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; CCR, CC chemokine receptor; MDC, macrophage-derived chemokine; PAS, periodic acid-Schiff; RPA, RNase protection assay; Stat, signal transducer and activation of transcription; TARC, thymus- and activation-regulated chemokine; TCA, thymus-derived chemotactic agent; VCAM, vascular cell adhesion molecule.

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nary eosinophilia, AHR, and mucus production were decreased in Stat6$^{-/-}$ mice that were sensitized and challenged with OVA compared with their wild-type littermates (16–18). Stat6$^{-/-}$ mice also do not develop Th2 cytokine– and allergen-specific IgE responses in this model, indicating a critical role for Stat6 in mediating the allergic airway response by inducing Th2 cell differentiation. However, the marked defect in Th2 cell differentiation in these mice has precluded these studies from addressing the role of Stat6 in the trafficking and effector functions of Th2 cells in the allergic lung.

To understand the role of Stat6 in Th2 cell trafficking and Th2 cell effector functions in vivo apart from its role in Th2 differentiation, we adoptively transferred OVA-specific (Stat6$^{+/+}$) Th2 cells into Stat6$^{-/-}$ and Stat6$^{+/+}$ mice followed by OVA aerosol challenges. We found that the ability of adoptively transferred Th2 cells to mediate the asthma phenotype, including pulmonary eosinophilia, mucus production, and AHR, was dependent on Stat6 expression in resident cells of the lung. Our results demonstrate a novel role for Stat6 in the recruitment of Th2 cells and eosinophils to the lung and the production of chemokines active on these cells.

Materials and Methods

Mice. DO11.10 mice transgenic for the TCR recognizing OVA peptide 323–339 (pOVA$_{323–339}$) were bred in our facility. Stat6$^{-/-}$ and wild-type Stat6$^{+/+}$ mice on a Balb/c background between 7 and 9 wk of age were obtained from The Jackson Laboratory.

Generation of Th2 Cells. CD4 T cells were isolated from spleen and pooled LNs of D011.10 mice using Dynal beads. Purified CD4 T cells (2×10^5 cells/ml) were activated in the presence of 50 μg/ml mitomycin c–treated Balb/c splenocytes (2×10^6 cells/ml), 1 μg/ml pOVA$_{323–339}$, 1,000 U/ml IL-4 (PeproTech), anti–IFN-γ (R46A2) at inhibitory concentrations, and anti-CD28 at stimulatory concentrations in 24-well plates. On days 3 and 5, cells were fed with 5–10 U/ml of IL-2 (PeproTech). At the time of transfer, an aliquot of cells was retained for restimulation. 1×10^6 CD4 cells and 2×10^6 freshly isolated mitomycin c–treated Balb/c splenocytes were cultured with 1 μg/ml pOVA$_{323–339}$. Supernatants were collected at 48 h after stimulation for cytokine ELISA. Th2 cells produced between 50 and 150 ng/ml of IL-4, 50 and 150 ng/ml of IL-5, 30 and 75 ng/ml of IL-13, and very low levels of IFN-γ.

Transfer of Th2 Cells and OVA Challenges. Th2 cells were harvested on days 5 and 6, washed twice in PBS, and 5×10^6 cells were injected intravenously into Stat6$^{+/+}$ or Stat6$^{-/-}$ Balb/c recipients. After transfer, mice were challenged seven times (for 20 min daily) with a 5% OVA solution or PBS (control mice) using a nebulizer (Pulmo Aide; DeVil Biss).

Determination of AHR. AHR was measured in Stat6$^{+/+}$ and Stat6$^{-/-}$ mice after six OVA or PBS aerosol challenges by recording respiratory pressure curves using whole body plethysmograph (Buxco; EMKA Technologies) in response to inhaled methacholine (Sigma-Aldrich) at concentrations ranging from 3 to 50 mg/ml. AHR was expressed in enhanced pause (P_{enh}), a calculated value that correlates with measurement of airway resistance, impedance, and intrapleural pressure in the same mouse.

BAL and LN Isolation. BAL was performed ~18 h after the last aerosol challenge with six 0.5-ml aliquots of PBS containing 0.6 mM EDTA. BAL cytokine levels were measured using the lavage fluid recovered from the first 1 ml of instilled PBS/EDTA to avoid overdilution of the BAL fluid components. Recovered live cells (Trypan blue exclusion) were enumerated using a hemocytometer. Cell differential counts were determined on DiffQuik-stained cytocentrifuge preparations of cells recovered from BAL. Single cell suspensions of paratracheal LN cells were prepared for flow cytometry analysis.

Histology, Northern Blot Analysis, and RNase Protection Assays. Lungs were harvested after the BAL and inflation was fixed to total lung capacity in 10% formalin. Formalin-preserved lung tissue was stained with hematoxylin and eosin or diastase periodic acid-Schiff (PAS). The severity of inflammation was graded semiquantitatively for the following features: eosinophils, lymphocytes, granulomatous inflammation, giant cells, macrophages, and mucous cells. Each of these features was graded on a scale of 0 to 4 where 0 = none, 1 = mild, 2 = moderate, 3 = severe, and 4 = very severe based on the severity and extent of the features in the histological sections. Randomized and blinded slides were graded by F. Green. For RNA isolation, lungs were snap frozen in liquid nitrogen and lysed in guanidium hydrochloride followed by pelleting through a $CsCl_2$ gradient. For the RNase protection assay (RPA), 5 μg of RNA from each sample was analyzed using Riboquant RPA kits mCK-1 (BD PharMingen). For Northern blot analysis, 20 μg of RNA from each sample was analyzed as described previously (19). Murine cDNA probes for macrophage-derived chemokine (MDC), thymus- and activation-regulated chemokine (TARC) (ABCD-2), and thymus-derived chemotactic agent (TCA)-3 were gifts from P. Gray (ICOS Corp., Bethell, WA), C. Schaniel (Basel Institute of Immunology, Boston, MA), and M. Dorf (Harvard Medical School, Boston, MA), respectively.

Cytokine Assays. Levels of IL-4, IL-5, and IFN-γ were measured by ELISA (Endogen) as were levels of IL-13 (R&D Systems).

Flow Cytometry. Cell suspensions from the BAL and LNs were analyzed by three-color flow cytometry on a FACScan™ (Becton Dickinson) cytofluorimeter as described previously (19). Commercial conjugated antibodies used for cell staining included: anti-CD4–PE; anti-CD3–FITC; anti-CD8–CyChrome; anti-CD25–FITC; anti-B220–PE (BD PharMingen); and anti-KJ1-26–FITC (Caltag Biochemicals), an antibody specific for the transgenic TCR in the DO11.10 mice.

Statistical Analysis. Student's *t* test (unpaired, two-tailed) was used to calculate significance levels for all measurements except lung histology scores, which were compared using Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

Stat6 Is Essential for Airway Eosinophilic Inflammation after Transfer of Stat6$^{+/+}$ Th2 Cells and Aerosol Challenge. Studies using Stat6$^{-/-}$ mice have revealed that Stat6 plays an essential role in the development of allergic inflammation (16–18). Since Stat6$^{-/-}$ mice do not develop Th2 cells, the role of Stat6 in Th2 cell trafficking could not be assessed in these experiments. We generated OVA-specific Stat6$^{+/+}$ Th2 cells from DO11.10 mice in vitro using OVA peptide$_{323–339}$, IL-4, and anti–IFN-γ. We confirmed that these cells were functional Th2 cells by their preferential

secretion of high levels of IL-4, IL-5, and IL-13 and very low levels of IFN-γ. To determine if Stat6 is involved in trafficking of Th2 cells into the lung, we transferred OVA-specific Stat6$^{+/+}$ Th2 cells into Stat6$^{+/+}$ and Stat6$^{-/-}$ mice by tail vein injection followed by aerosol challenges with OVA or PBS. After adoptive transfer and aerosol OVA challenge, the total number of cells recovered from the BAL of Stat6$^{-/-}$ mice was significantly reduced compared with cells recovered from the BAL of Stat6$^{+/+}$ mice (1.6×10^6 vs. 5.9×10^6 cells; Fig. 1 a). As expected, 50–60% of BAL cells in OVA-challenged Stat6$^{+/+}$ (OVA-Stat6$^{+/+}$) mice were eosinophils. In striking contrast, only 1–2% of BAL cells in OVA-Stat6$^{-/-}$ mice were eosinophils (Fig. 1 a). The effect of Stat6 on the accumulation of other cell types was more modest with OVA-Stat6$^{-/-}$ mice having a 50% reduction in lymphocytes, a 30% reduction in monocytes, and no significant differences in the number of neutrophils compared with OVA-Stat6$^{+/+}$ mice. The few cells recovered from the BAL of PBS-challenged Stat6$^{+/+}$ and Stat6$^{-/-}$ mice were almost entirely macrophages (96–99%; Fig. 1 a). Thus, adoptive transfer of Stat6$^{+/+}$ Th2 cells was insufficient to recruit eosinophils into the BAL in Stat6$^{-/-}$

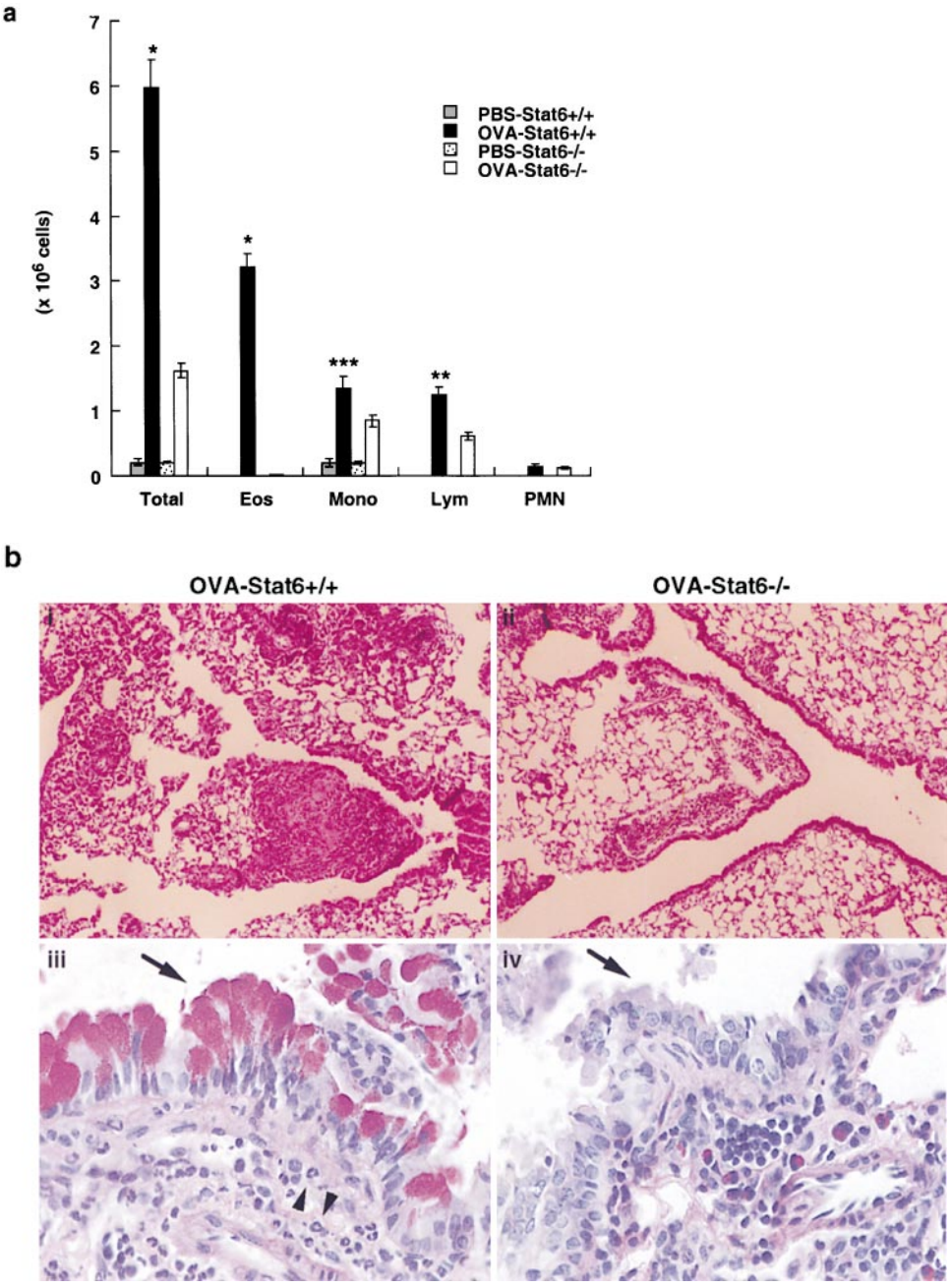

Figure 1. Attenuated eosinophilic airway inflammation and mucus cell production in Stat6$^{-/-}$ mice after Th2 cell transfer and aerosol OVA challenge. (a) BAL cell counts. OVA-specific Stat6$^{+/+}$ Th2 cells were transferred intravenously into Stat6$^{+/+}$ and Stat6$^{-/-}$ mice and exposed to daily challenges with aerosolized OVA or PBS for 7 d. 18 h after the last OVA challenge, leukocytes were recovered from the BAL and total and differential counts were performed. Data represent mean number of BAL cells (± SEM; n = 10 for OVA-Stat6$^{+/+}$ and OVA-Stat6$^{-/-}$ mice; n = 3 for PBS-Stat6$^{+/+}$ and PBS-Stat6$^{-/-}$ mice). *$P < 0.0001$; **$P = 0.0003$; and ***$P = 0.02$ in OVA-Stat6$^{+/+}$ versus OVA-Stat6$^{-/-}$ mice. (b) Hematoxylin and eosin (i and ii) and PAS (iii and iv) stained formalin-fixed lung sections isolated from Stat6$^{+/+}$ (i and iii) and Stat6$^{-/-}$ mice (ii and iv) after transfer of OVA-specific Th2 cell and aerosol OVA challenge described above. (i) Stat6$^{+/+}$. Characteristic intense peribronchial inflammatory cell infiltrate comprised of lymphocytes and eosinophils. Enlarged multinucleated giant cells are seen within the inflammation and the alveolar spaces. In the peribronchial location, they are loosely organized into a granuloma. (ii) Stat6$^{-/-}$. By contrast, Stat6$^{-/-}$ mice show a marked attenuated inflammatory response around the airways with notable and marked decreases in eosinophils. There is almost complete absence of multinucleated giant cells and granulomas. (iii and iv) PAS staining of lung sections revealed characteristic mucin staining in bronchial epithelium (arrow) of Stat6$^{+/+}$ (iii) mice, which is almost completely absent in Stat6$^{-/-}$ mice (iv). Similarly treated PBS-challenged Stat6$^{+/+}$ and Stat6$^{-/-}$ had no pulmonary inflammation or mucus production (data not shown). Note presence of subepithelial eosinophils (arrowheads) in Stat6$^{+/+}$ (iii) but not Stat6$^{-/-}$ (iv) mice. Original magnifications: (i and ii) ×125; (iii and iv) ×600.

Table I. *Histopathologic Features in Lungs of OVA-Stat6$^{+/+}$ and OVA-Stat6$^{-/-}$ Mice after Adoptive Transfer of Th2 Cells*

	Eosinophils in					
	Venules	Bronchi	Alveoli	Giant cells	Granulomas	Mucous cells
OVA-Stat6$^{+/+}$	3+	3+	2	3+	2	3+
OVA-Stat6$^{-/-}$	1+	1+	0	0	0	1
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

OVA-specific Th2 cells were transferred into Stat6$^{+/+}$ and Stat6$^{-/-}$ mice and exposed to daily aerosol OVA challenges for 7 d. 18 h after the last challenge, lungs were harvested, formalin fixed, and stained with hematoxylin and eosin or diastase PAS. The extent of the inflammatory cell infiltrate was graded in a randomized and blinded fashion on a scale of 0 to 4. Data represent the average of 10 mice per group.

mice after aeroallergen challenge, indicating that Stat6 signaling in the lung is necessary for eosinophil recruitment.

Airway Eosinophilic Inflammation and Goblet Cell Mucus Production Are Reduced in the Lungs of Stat6$^{-/-}$ Mice. Histopathologic examination of lung tissue revealed that the cardinal features of allergic inflammation were absent in the Stat6$^{-/-}$ mice after Th2 cell transfer and OVA challenge. Semiquantitative analysis revealed OVA-Stat6$^{+/+}$ mice had peribronchial and perivascular infiltrates consisting mainly of eosinophils, lymphocytes, multinucleated giant cells, and granulomas. In contrast, OVA-Stat6$^{-/-}$ mice had markedly diminished eosinophil accumulation in these locations, similar lymphocyte accumulation, and a virtual absence of multinucleated giant cells and granulomas (Table I). Similarly, OVA-challenged Stat6$^{+/+}$ mice had many cells in the airway epithelium that stained positive for mucus (Fig. 1 b, iii). In contrast, very few cells were positive for mucus production in OVA-Stat6$^{-/-}$ mice (Fig. 1 b, iv, and Table I), which was similar to what we detected in the PBS-treated controls (data not shown). Our data reveal a critical role for Stat6 signaling in mediating the ability of Th2 cells to induce eosinophilic inflammation, multinucleated giant cell and granuloma formation, and mucus production in the lungs of antigen-challenged mice in vivo.

Antigen-induced AHR Is Decreased in Stat6$^{-/-}$ Mice. The physiology of asthma is characterized by AHR which is also regulated by Th2 cells. OVA-Stat6$^{+/+}$ mice developed significant increases in AHR at 6.25 and 12.5 mg/ml of methacholine, whereas OVA-Stat6$^{-/-}$ mice had no significant increases in AHR over PBS-challenged Stat6$^{+/+}$ and Stat6$^{-/-}$ mice (Fig. 2). At higher doses of methacholine, increases in AHR were seen in OVA-Stat6$^{-/-}$ mice compared with the PBS-challenged mice but the magnitude was lower than seen in the OVA-challenged Stat6$^{+/+}$ mice (Fig. 2). Thus, Stat6 expression in the resident cells of the lung is also required for the induction of antigen-induced AHR by Th2 cells in allergic inflammation.

OVA-specific Th2 Cells Are Markedly Decreased in the BAL of OVA-Stat6$^{-/-}$ Mice. To determine if the failure of transferred Th2 cells to mediate the asthma phenotype in Stat6$^{-/-}$ mice resulted from an inability of these cells to traffic to the airways, we analyzed the recruitment of transferred OVA-specific CD4$^+$ cells into the BAL and paratracheal LNs. Aerosol OVA challenge resulted in a large influx of lymphocytes into the BAL of OVA-Stat6$^{+/+}$ mice (1.2×10^6 cells), while PBS-challenged Stat6$^{+/+}$ mice had essentially no lymphocytes in the BAL (Fig. 1 a). Of the lymphocytes recovered from the BAL of OVA-Stat6$^{+/+}$ mice, 46% of the cells were CD4$^+$ (56×10^4 cells) and 16% (19×10^4 cells) were OVA-specific (Fig. 3, a and b). In striking contrast, 18% of the BAL lymphocytes in OVA-Stat6$^{-/-}$ mice were CD4$^+$ (12×10^4 cells) and only 1.7% (1.1×10^4 cells) were OVA-specific (Fig 3, a and b). The BAL of OVA-Stat6$^{+/+}$ mice had an increased percentage of activated CD4$^+$ (CD25$^+$) cells (8.0 vs. 3%) and a decrease in the percentage of B cells (B220$^+$) (9.1 vs. 12%) and CD8$^+$ cells (14 vs. 23%) compared with OVA-Stat6$^{-/-}$ mice (Table II).

In paratracheal LNs, the total number of cells obtained from both OVA-Stat6$^{+/+}$ and OVA-Stat6$^{-/-}$ mice was highly increased compared with their PBS-challenged

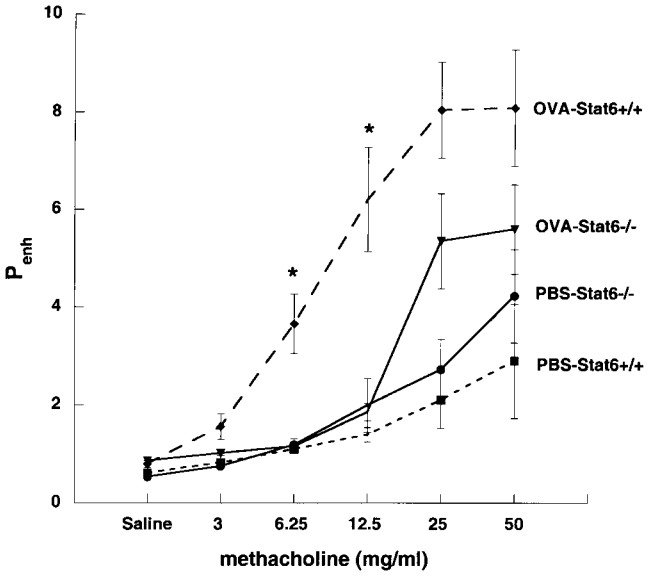

Figure 2. Stat6$^{-/-}$ mice have attenuated AHR after transfer of Th2 cells and OVA challenge. Airway reactivity to increasing concentrations of methacholine was measured in OVA-Stat6$^{+/+}$ ($n = 8$), OVA-Stat6$^{-/-}$ ($n = 8$), PBS-Stat6$^{+/+}$ ($n = 4$), and PBS-Stat6$^{-/-}$ ($n = 4$) mice. Data represent mean P_{enh} values (± SEM). *$P < 0.001$ in OVA-Stat6$^{+/+}$ versus OVA-Stat6$^{-/-}$ mice.

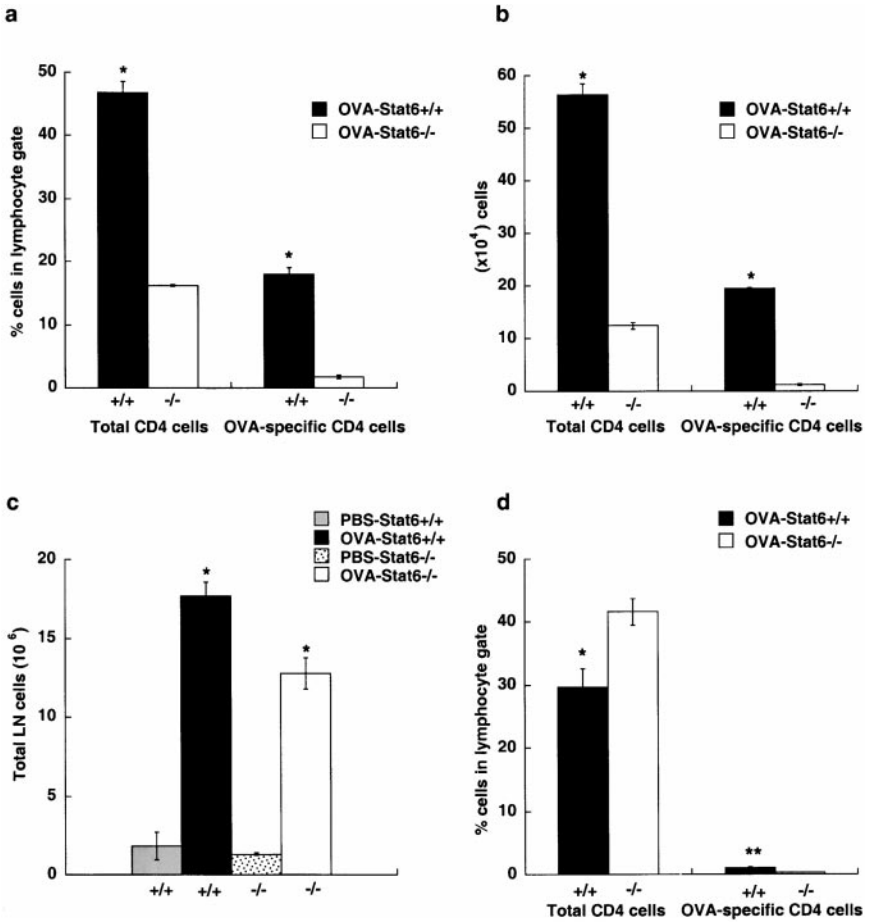

Figure 3. Decreased recruitment of OVA-specific Th2 cells in the BAL despite activation in paratracheal LNs of Stat6$^{-/-}$ mice. (a and b) Decreased OVA-specific CD4$^+$ cells in the BAL of OVA-Stat6$^{-/-}$ mice. FACS® analysis was performed on BAL cells isolated from mice described above. Cells were stained with anti-CD4 and KJ1-26 (OVA-TCR specific) antibodies. (a) Percentage of CD4 and OVA-specific CD4 cells in the BAL of Stat6$^{+/+}$ and Stat6$^{-/-}$ mice. *$P < 0.0001$ in OVA-Stat6$^{+/+}$ versus OVA-Stat6$^{-/-}$ mice. (b) Total number of OVA-specific cells in the BAL = (% KJ$^+$ cells in the lymphocyte gate) × (total number of lymphocytes calculated by differential counts). *$P < 0.0001$ in OVA-Stat6$^{+/+}$ versus OVA-Stat6$^{-/-}$ mice. (c and d) Analysis of paratracheal LNs of OVA- and PBS-challenged Stat6$^{+/+}$and Stat6$^{-/-}$ mice after Th2 cell transfers. (c) Total LN cells recovered from paratracheal LNs (n = 10 for OVA-Stat6$^{+/+}$ and OVA-Stat6$^{-/-}$ mice; n = 3 for PBS-Stat6$^{+/+}$ and PBS-Stat6$^{-/-}$ mice). *$P < 0.0001$ in OVA-Stat6$^{+/+}$ versus PBS-Stat6$^{+/+}$ mice and OVA-Stat6$^{-/-}$ versus PBS-Stat6$^{-/-}$ mice. (d) Percentage of CD4 and OVA-specific cells in paratracheal LNs (mean of five mice/group, representative of two experiments). There were too few CD4$^+$ cells in the BAL of PBS-Stat6$^{+/+}$ and PBS-Stat6$^{-/-}$ mice for FACS® analysis. *$P = 0.004$ in Stat6$^{+/+}$ versus Stat6$^{-/-}$ mice; **$P < 0.0001$ in Stat6$^{+/+}$ versus Stat6$^{-/-}$ mice.

counterparts (Fig. 3 c), and compared with OVA-challenged mice without adoptive transfer (data not shown). The percentage of CD4$^+$ cells in the LNs of Stat6$^{-/-}$ was increased (42 vs. 30%), whereas the percentage of OVA-specific T cells was decreased (0.3 vs. 1%) compared with OVA-Stat6$^{+/+}$ mice (Fig. 3 d). There were no significant differences between B cells and CD8$^+$ cell populations in the LNs of the two groups (Table II). These data demonstrate that despite evidence of antigen activation of transferred Th2 cells in the paratracheal LNs, trafficking of transferred OVA-specific Th2 cells to the airways of Stat6$^{-/-}$ mice was dramatically impaired.

Table II. *Phenotype of BAL and LN Lymphocytes in OVA-Stat6$^{+/+}$ and OVA-Stat6$^{-/-}$ Mice after Adoptive Transfer of Th2 Cells*

		Percentage of cells in lymphocyte gate				
	Source of cells	B220$^+$	CD3$^+$	CD4$^+$	CD4$^+$CD25$^+$	CD8$^+$
OVA-Stat6$^{+/+}$	BAL	9	69	49	8	14
OVA-Stat6$^{-/-}$	BAL	12	50	20	3	23
P value		NS	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.01$
OVA-Stat6$^{+/+}$	Draining LNs	23	75	46	3	22
OVA-Stat6$^{-/-}$	Draining LNs	21	74	55	3	18
P value		NS	NS	$P < 0.001$	NS	$P = 0.02$

OVA-specific Stat6$^{+/+}$ Th2 cells were transferred into Stat6$^{+/+}$ and Stat6$^{-/-}$ mice followed by daily OVA aerosol challenges for 7 d. FACS® analysis was performed on single cell suspensions of paratracheal LNs and BAL cells with the indicated antibodies. Values represent mean percentage of cells in the lymphocyte gate for the individual mice (n = 5 mice per group). Data are representative of two independent experiments. Similarly treated PBS-challenged mice had too few CD4$^+$ T cells in the BAL and paratracheal LN cells for these studies.

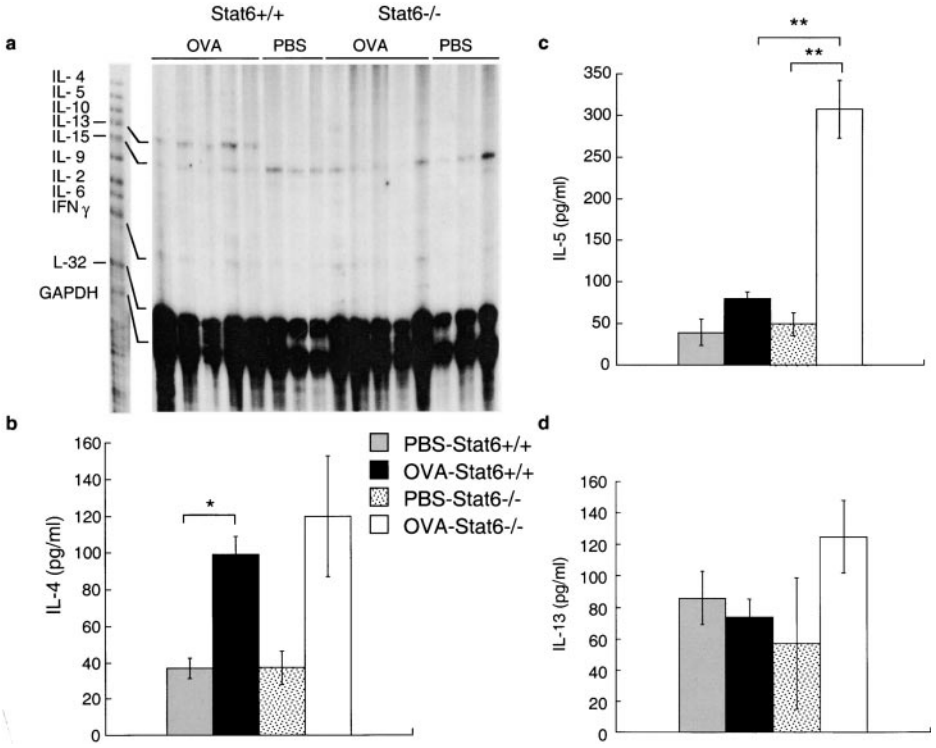

Figure 4. Cytokine levels in the lung and BAL of Stat6$^{+/+}$ and Stat6$^{-/-}$ mice. (a) Cytokine mRNA expression was determined by RPA analysis. Each lane represents a single mouse. Levels of IL-4 (b), IL-5 (c), and IL-13 (d) were measured in the BAL of OVA- and PBS-challenged Stat6$^{+/+}$ and Stat6$^{-/-}$ mice by ELISA. Data are presented as mean cytokine level (± SEM; $n = 5$ mice for IL-4 and IL-5; $n = 10$ for IL-13; $n = 3$ for all PBS-challenged controls). *$P = 0.004$ in OVA-Stat6$^{+/+}$ versus PBS-Stat6$^{+/+}$ mice; **$P = 0.0002$ in OVA-Stat6$^{+/+}$ versus OVA-Stat6$^{-/-}$ mice; and $P = 0.001$ in OVA-Stat6$^{-/-}$ versus PBS-Stat6$^{-/-}$ mice. No significant differences were detected in IL-13 levels.

Allergen-induced BAL and Lung Cytokine Profile in Stat6$^{+/+}$ and Stat6$^{-/-}$ Mice. As Th2 cell cytokines are important in regulating allergic inflammation, we determined levels of IL-4, IL-5, and IL-13 mRNA in the lung and protein in the BAL. Increased levels of IL-13 mRNA were detected in the lungs of OVA-Stat6$^{+/+}$ mice, but not in the lungs of the other groups (Fig. 4 a). Levels of IFN-γ and IL-15 were detected in all groups with no elevations in antigen-challenged mice. Elevated levels of IL-4 were detected in the BAL of OVA-challenged mice compared with PBS-challenged controls with no differences between antigen-challenged Stat6$^{+/+}$ and Stat6$^{-/-}$ mice (Fig. 4 b). Levels of IL-5 were surprisingly higher in OVA-Stat6$^{-/-}$ compared with Stat6$^{+/+}$ mice (307 vs. 80 pg/ml). We speculate that this difference results from the increased consumption of IL-5 in the BAL of Stat6$^{+/+}$ mice secondary to the massive

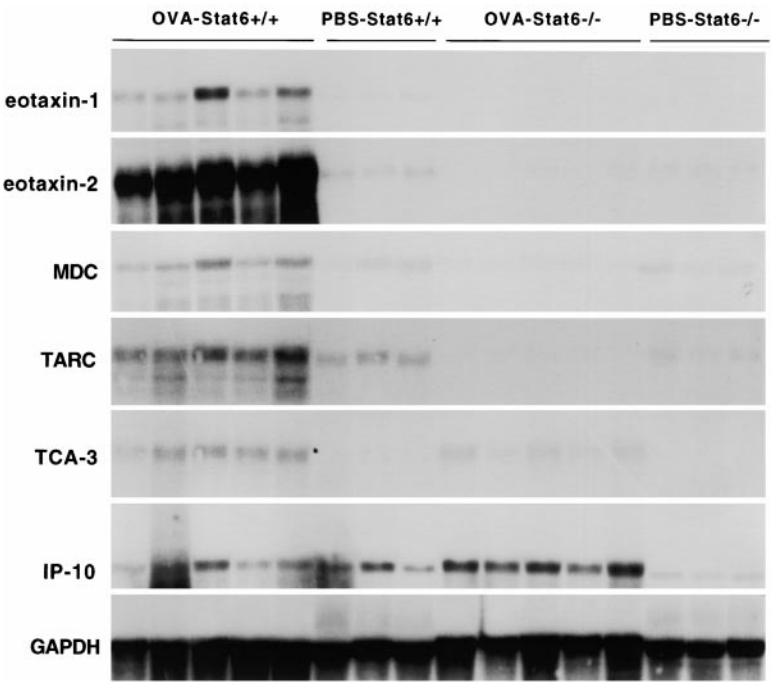

Figure 5. Chemokine induction in the lungs of OVA- and PBS-challenged Stat6$^{+/+}$ and Stat6$^{-/-}$ mice. Chemokine mRNA expression was determined by Northern blot analysis. Each lane contains RNA from a different mouse. Blots were sequentially hybridized with the cDNA probes for the indicated chemokine and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control for RNA loading. Blots were exposed for 72 h except for TCA-3, which was exposed for 2 wk.

influx of pulmonary eosinophilia which is absent in the BAL of Stat6$^{-/-}$ mice. In contrast to the RNA data, we detected no significant differences in IL-13 protein levels in the BAL of OVA- and PBS-challenged Stat6$^{+/+}$ and Stat6$^{-/-}$ mice (Fig. 4 d). The difference in IL-13 profile in BAL and lung that we observed may reflect differential regulation of cytokine synthesis in these two compartments of the respiratory tract. In addition, no significant differences in IFN-γ levels were detected between the groups (data not shown). Since the primary source of IL-4, IL-5, and IL-13 is likely to be Th2 cells, the results indicate that the very small percentage of OVA-specific T cells that were recruited to the BAL of Stat6$^{-/-}$ mice were functional.

Differential Chemokine Gene Expression in the Lungs of Stat6$^{+/+}$ and Stat6$^{-/-}$ Mice. The predominant chemokine receptor expressed on eosinophils is CC chemokine receptor (CCR)3 (20), whereas Th2 cells express CCR3, CCR4, and CCR8 (21). We found highly elevated levels of the CCR3 agonists eotaxin (CCL11) and eotaxin-2 (CCL24), and the CCR4 agonists MDC (CCL22) and TARC (ABCD-2/CCL17) in the lungs of OVA-Stat6$^{+/+}$ mice compared with all other groups (Fig. 5). Our analysis also revealed that expression of the CCR8 agonist, TCA-3 (I-309/CCL1), was markedly increased in the lungs of OVA-Stat6$^{+/+}$ mice with lower expression in the lungs of OVA-Stat6$^{-/-}$ mice. TCA-3 is a chemokine secreted by activated T cells, preferentially of the Th2 type (22, 23). The very faint levels of TCA-3 detected in OVA-Stat6$^{-/-}$ mice may simply reflect the decreased numbers of activated T cells found in the lungs of the Stat6$^{-/-}$ mice. In contrast to the expression of these "Th2-type" chemokines, the expression of IP-10, an IFN-γ–inducible Th1-type chemokine active on Th1 cells, was significantly elevated in OVA-Stat6$^{-/-}$ mice compared with PBS-challenged controls. These results demonstrate that Stat6 is essential for the induction of chemokines critical for eosinophil and Th2 cell recruitment to the allergic lung.

Discussion

Using an adoptive transfer model, we have demonstrated that recruitment of antigen-specific Th2 cells to the airways is Stat6 dependent. The reduction in Th2 cell accumulation in the airways of OVA-Stat6$^{-/-}$ mice was accompanied by a dramatic decrease in eosinophilia, goblet cell mucus production, and AHR. The expression of key chemokines implicated in Th2 cell and eosinophil trafficking, such as eotaxin, eotaxin-2, TCA-3, TARC, and MDC, was markedly attenuated in the lungs of OVA-Stat6$^{-/-}$ mice. Therefore, our data demonstrate that in addition to its well-established role in Th2 cell differentiation, Stat6 plays an important role in Th2 cell recruitment, Th2 type chemokine expression, and Th2 cell effector function in vivo.

Airway eosinophilia is one of the characteristic features of asthma that was absent in the OVA-Stat6$^{-/-}$ mice despite the adoptive transfer of functional Th2 cells. Eosinophils migrate from the vasculature into different compartments of the allergic lung where they damage the airway epithelial cells that line the respiratory tract (24, 25). IL-5 has been clearly defined as the critical factor needed for the differentiation and activation of eosinophils (26). Previous studies have shown that eosinophilia is markedly diminished in Stat6$^{-/-}$ mice that have been sensitized and challenged with OVA. In one study, reconstitution of Stat6$^{-/-}$ mice with IL-5 during OVA challenge resulted in a marked increase in eosinophilia, highlighting the importance of IL-5 in this process (18). However, in our study eosinophilia was dramatically decreased in the BAL and lungs despite elevated levels of IL-5 in the BAL of antigen-challenged Stat6$^{-/-}$ mice, demonstrating that Stat6-dependent factors are necessary to recruit eosinophils in response to Th2 cytokines.

Secretion of mucus glycoproteins from the goblet cells of the airway epithelium is another characteristic feature of the asthma phenotype (1). We found that this feature of asthma was also absent in Stat6$^{-/-}$ mice after OVA-specific Th2 cell transfer and OVA challenge. The regulation of mucus hypersecretion is under the control of Th2 cytokines (1). In fact, overexpression of IL-4 in transgenic mice induces increased mucus cell production and MUC 5 gene expression from the epithelium (27). However, despite comparably elevated levels of IL-4 in the BAL of OVA-Stat6$^{-/-}$ and OVA-Stat6$^{+/+}$ mice, mucus hypersecretion was absent in the lungs of OVA-Stat6$^{-/-}$ mice. Therefore, our data suggest that in addition to its requirement for IL-4 production, Stat6 signaling is required for the airway epithelium to respond to IL-4 with increased mucus production.

Histopathological analysis also revealed a dramatic decrease in pulmonary granuloma and multinucleated giant cell formation after adoptive transfer and OVA challenge in Stat6$^{-/-}$ mice. Granulomatous inflammation has been described in this murine model and likely represents a pathological response to inhaled protein antigen. Little is known about the molecular signals that regulate giant cell and granuloma formation, although it has been reported that IL-4 can induce giant cell formation from macrophages in vitro (28). Our data indicate that Stat6 signaling is necessary for the formation of giant cells and granulomas in this in vivo model.

AHR is the third hallmark of asthma induced by the Th2-type inflammation in the airways (1) that was absent in the OVA-Stat6$^{-/-}$ mice. Although the precise mechanism is poorly understood, recent studies suggest an important role for Th2 cytokines, such as IL-4, IL-5, and IL-13 in AHR (1). Genetic deficiency or neutralization of each of these cytokines diminished antigen-induced AHR in vivo, whereas overexpression of IL-5 and IL-13 resulted in increased AHR at baseline (5, 6, 29, 30). In fact, in a study mentioned above, reconstitution of Stat6$^{-/-}$ mice with IL-5 during OVA challenge resulted in a marked increase in AHR (18). In our studies, adoptive transfer of Th2 cells into OVA-Stat6$^{-/-}$ mice failed to induce AHR despite elevated levels of IL-4 and IL-5 in the BAL. This finding could be explained either by the failure of the airway epi-

thelium to respond to IL-4 and IL-5 due to the lack of Stat6 signaling or by decreased IL-13 levels as seen by RPA in the OVA-STAT6$^{-/-}$ mice.

Th2 cells adoptively transferred into Stat6$^{-/-}$ mice failed to traffic to the lung after OVA challenge. The molecular mechanisms regulating Th2 cell trafficking to the allergic lung are not known (31). Possible mechanisms regulating Th2 cell trafficking include selective expression of adhesion molecules and their counter ligands or selective expression of chemokine receptors and Th2-active chemokines. Th1 cells express high levels of functional selectin ligands, such as P-selectin ligand on their surface, which enable them to be preferentially recruited to the sites of Th1 inflammation (32–34). These cells also preferentially express CXC chemokine receptor (CXCR)3 and CCR5 and can be selectively recruited by Th1-type chemokines, such as IP-10. However, it is not clear whether Th2 cells express selective adhesion molecules that facilitate their entry into the allergic lung. The interaction between very late antigen 4 on Th2 cells and vascular cell adhesion molecule (VCAM)-1 on the inflamed endothelium has been proposed as an attractive candidate to mediate selective recruitment of Th2 cells in allergic inflammation as IL-4 upregulates VCAM-1 on endothelial cells (35). However, Stat6 is not involved in the upregulation of VCAM-1 on pulmonary endothelial cells, as VCAM-1 expression was increased in both Stat6$^{+/+}$ and Stat6$^{-/-}$ mice that were sensitized and challenged with OVA, making differences in VCAM-1 expression an unlikely explanation for our result (17). In the absence of a specific molecular mechanism to explain Th2 cell–specific recruitment, it has been proposed that Th2 cells passively accumulate in allergic tissues as a result of the increased vasodilation seen during asthma (31).

However, Th2 cells preferentially express CCR3, CCR4, and CCR8, and chemokines active on these receptors are elevated in allergic inflammation, making them attractive candidates to mediate Th2 cell–specific recruitment (21). Inhibition or deletion of single chemokines or chemokine receptors (e.g., eotaxin, MDC, CCR3, CCR4) have not abrogated Th2 cell recruitment in allergic pulmonary inflammation (36–39). We hypothesized that multiple and/or additional chemokines control Th2 cell trafficking in this model, preserving trafficking of these cells in the absence of individual chemokines or their receptors. We found a global deficiency of chemokines active on Th2 cells and eosinophils in Stat6$^{-/-}$ mice, which could explain the marked reduction in Th2 cell and eosinophil trafficking seen in our study. Therefore, our data provide the first evidence that there are specific molecular signals that guide Th2 cell trafficking and implicate Stat6-dependent chemokines in this process.

We hypothesized that Th2 cytokines induce chemokine production by resident parenchymal cells of the lung via Stat6 signaling. IL-4 and IL-13 induce eotaxin-1, -2, and -3 expression in airway epithelial cells and endothelial cells in vitro and in vivo (40–45), and IL-4 induction of eotaxin-1 gene expression was found to be Stat6 dependent (46). IL-4 also induces MDC and TARC expression in macrophages and respiratory epithelial cells and these chemokines have been implicated in allergic inflammation (37, 47). We have shown that eotaxin-1, eotaxin-2, MDC, TARC, and TCA-3 expression in the allergic lung in vivo is Stat6 dependent. Our finding that Stat6 signaling is necessary for the production of all Th2 active chemokines is consistent with there being functional Stat6 binding sites in the promoters for these chemokines. While our study does not establish the specific cellular source of Stat6-dependent chemokine production, our adoptive transfer experiments demonstrate that the expression of these chemokines requires Stat6 signaling in resident cells of the lung. Therefore, our data suggest that Stat6-inducible chemokines are the molecular link between antigen-induced Th2 cell activation and Th2 cell and eosinophil recruitment in vivo.

The initial trafficking of Th2 cells to the airways in our model is Stat6 independent since we were able to detect a few OVA-specific Th2 cells in the BAL of Stat6$^{-/-}$ mice. It is possible that these Th2 cells accumulate in the BAL "passively" due to pulmonary vasodilation or that they are actively recruited by Stat6-independent chemokine production in the lungs after OVA aerosol challenges. The ultimate localization of Th2 effector cells into the lung, however, was markedly impaired in Stat6$^{-/-}$ mice, despite evidence of antigen activation of transferred Th2 cells in the paratracheal LNs of these mice. These results demonstrate that trafficking of Th2 cells to the lung is an active process dependent on Stat6 signaling in resident parenchymal cells of the lung.

Our data support a model in which the initial aerosol OVA challenge allows limited numbers of OVA-specific Th2 cells to enter the lung in a Stat6-independent process. Once in the lung these Th2 cells secrete cytokines, such as IL-4 and IL-13, that activate resident pulmonary cells in a Stat6-dependent manner to secrete chemokines active on Th2 cells and eosinophils. These chemokines then amplify the Th2 response by attracting large numbers of Th2 cell and eosinophils. Recruited Th2 cells then induce mucus production and AHR in a Stat6-dependent manner.

Overall our findings establish that Stat6 is a master regulator of allergic inflammation. In addition to its known ability to control Th2 cell generation through its activity in lymphocytes, we have now shown that Stat6 also controls Th2 cell trafficking through its activity in resident cells of the lung.

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