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**Cutting Edge: Th2 Cell Trafficking into the Allergic Lung Is Dependent on Chemoattractant Receptor Signaling**

Anuja Mathew,* Benjamin D. Medoff,*† Andrew D. Carafone,* and Andrew D. Luster**

Th2 cells are recruited to the lung where they mediate the asthma phenotype. Since the molecular mechanisms regulating Th2 cell trafficking remain unknown, we sought to determine whether trafficking of Th2 cells into the lung is mediated by Gαi-coupled chemoattractant receptors. We show here that in contrast to untreated Th2 cells, pertussis toxin-treated Th2 cells were unable to traffic into the lung, airways, or lymph nodes following Ag challenge and therefore were unable to induce allergic inflammation in vivo. Pertussis toxin-treated Th2 cells were functional cells, however, and when directly instilled into the airways of mice, bypassing their need to traffic to the lung, were able to induce airway eosinophilic inflammation. These studies conclusively demonstrate that trafficking of Th2 cells into the lung is an active process dependent on chemoattractant receptors. *The Journal of Immunology*, 2002, 169: 651–655.

T he CD4^+^ Th2 cell is a critical mediator of the asthma phenotype. Adoptive transfer of Th2 cells into naive mice followed by aerosol challenge has clearly demonstrated that Th2 cells are recruited into the lung and are critical for inducing the characteristic hallmarks of asthma, including airway hyperresponsiveness, eosinophilia, and mucus production (1). The mechanisms by which effector Th2 cells traffic into the allergic lung are unclear but chemoattractant receptors active on Th2 cells are prime candidates to mediate this process. Th2 cells have been shown to preferentially express the chemokine receptors CCR3, CCR4, CCR8, and the PGD<sub>2</sub> receptor CRTH2, while Th1 cells preferentially express CCR5, CXCR3, CXCR6, and CX<sub>3</sub>CR1 (2). This has led to the speculation that selective chemokine receptor expression on Th2 cells may enable these cells to preferentially migrate to the sites of inflammation in response to locally produced chemokines. However, targeted deletion of CCR3, CCR4, and CCR8 have not ablated Th2 cell trafficking in vivo, leaving the question open as to whether chemoattractant receptors expressed on Th2 cells control their trafficking in vivo (3–5).

We have recently shown that Stat6 expression in the lung plays a critical role in Th2 cell recruitment and Th2-type chemokine expression in allergic pulmonary inflammation (6). We have speculated that Th2 cell trafficking to the lung is dependent on the expression of Stat6-inducible chemokines by resident cells in the lung, implying that chemoattractant receptor activation on Th2 cells is responsible for their trafficking to the allergic lung. However, since other Stat6-inducible genes may also play a role in this process, we have not conclusively proven that Th2 cell trafficking into the lung is dependent on Stat6-inducible chemokine activation of chemoattractant receptors expressed on Th2 cells.

To determine whether chemoattractant receptor activation is required for Th2 cell trafficking into the lung, we pretreated Ag-specific Th2 cells with pertussis toxin (PTX), a known inhibitor of Gαi-coupled chemoattractant receptor-induced chemotaxis (7). We found that PTX-Th2 cells were unable to traffic into the lung in response to Ag challenge. PTX-Th2 cells were fully capable of secreting IL-4 and IL-5 however and were able to induce airway eosinophilic inflammation following Ag challenge when instilled directly into the airways. These studies demonstrate that chemoattractant receptors mediate the trafficking of Th2 cells into the lung during allergic inflammation.

**Materials and Methods**

**Generation of Th2 cells**

CD4^+^ T cells were isolated from spleen and pooled lymph nodes (LN)s of BALB/c mice and activated in the presence of mitomycin C-treated BALB/c splenocytes (APCs), OVA peptide 323–339 (OVAp), IL-4, anti-IFN-γ (R46A2), and anti-CD28 as previously described (6). On day 5, Th2 cells were resuspended at 4 × 10^5^ cells/ml and treated with PTX (100 ng/ml) for 24 h. An aliquot of cells was retained for FACS analysis, cytokine secretion, and proliferation assays. For cytokine secretion, 1 × 10^6^ Th2 or PTX-Th2 cells and 2 × 10^6^ freshly isolated APCs were cultured with OVAp (1 μg/ml) in 24-well plates. Supernatants were collected at 24–48 h after stimulation for ELISA. For the proliferation assays, indicated numbers of T cells were incubated with 2 × 10^5^ APCs and 1 μg/ml OVAp in 96-well plates. Wells were pulsed with 1 μCi [3H]TdR for 18 h on day 2 and was harvested on day 3, and [3H]TdR incorporation counted using a liquid scintillation counter.

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Transfer of Th2 cells and OVA challenges
Th2 cells or PTX-Th2 cells were harvested on day 6, washed twice in PBS, and 5 × 10^6 cells were injected i.v., using the tail vein, into BALB/c recipients. Mice were aerosol challenged for 20 min daily for 4 days with a 5% OVA solution using a nebulizer (Pulmo Aide; DeVil Biss, Somerset, PA). For the intratracheal instillations, mice were anesthetized, the trachea exposed, and 1 × 10^6 Th2 or PTX-Th2 cells in 80 μl of PBS were instilled directly into the trachea. The wound was closed using a 9-mm wound clip (Roboz Surgical Instruments, Rockville, MD) and removed before performing bronchoalveolar lavage (BAL). Mice were rested for a day and OVA aerosol-challenged for 20 min daily for 3 days.

BAL and lymphocyte isolation from organs
BAL was performed 18–24 h after the last aerosol challenge as described elsewhere (6). Lungs were minced and resuspended in PBS with 10% FBS, 850 U/ml hyaluronidase, and 150 U/ml collagenase A at 5°C. Lymphocytes were obtained from the lung, LNs, and spleens following passage through a cell strainer and RBC lysis.

Cytokine assays
Levels of IL-4 and IL-5 were measured by ELISA (Endogen, Woburn, MA).

Flow cytometry and histology
Cell suspensions from organs were analyzed by two-color flow cytometry using anti-CD4 PE, anti-LFA-1 PE, anti-very late activation Ag 4 (VLA-4) PE (BD Pharmingen, San Diego, CA), and anti-KJ1–26 FITC (Caltag Laboratories, Burlingame, CA), an Ab specific for the transgenic TCR in the D011.10 mice. The total number of KJ^+ cells was calculated by multiplying the (percent KJ^+ cells in the lymphocyte gate)/(total number of lymphocytes obtained from each of the organs). Lungs were harvested and inflation fixed to total lung capacity in 10% Formalin. Formalin-preserved lung tissue was stained with H&E or diastase periodic acid-Schiff stain.

Chemotaxis assays
Th2 or PTX-Th2 (treated with PTX for 2 h) cells were added (2.5 × 10^4 in 25 μl of RPMI 1640/1%BSA) into upper wells of a 5-μm filter 96-well ChemoTx plate (NeuroProbe, Gaithersburg, MD). Indicated concentrations of murine chemokines (PeproTech, Rocky Hill, NJ) were added in the bottom chamber in 31 μl of RPMI 1640/1% BSA. The plate was incubated for 1.5 h at 37°C in 5% CO2 and cells that migrated into the lower chamber were enumerated using a hemocytometer.

Statistical analysis
Student’s t test (unpaired, two tailed) was used to calculate significance levels for all measurements. A p < 0.05 was considered to be statistically significant.

Results
PTX treatment of Th2 cells does not alter cytokine secretion, integrin expression, or Ag-specific proliferation but blocks chemotaxis of Th2 cells in vitro
To ensure that PTX treatment did not alter other functions of Th2 cells, we compared cytokine secretion, in vitro proliferation and cell surface expression of key receptors on untreated Th2 and PTX-Th2 cells. We found that 3 days of PTX treatment did not alter the viability, secretion of IL-4 and IL-5, and did not affect Ag-induced proliferation (Fig. 1, A and B). Since integrins are known to be involved in cell migration, we also examined cell

![Figure 1](http://www.jimmunol.org/)

FIGURE 1. PTX does not alter cytokine secretion, proliferation, or cell surface receptor expression but blocks in vitro chemotaxis of Th2 cells (A). Cytokine secretion by Th2 and PTX-Th2 cells following restimulation in vitro with OVA-pulsed APCs. Supernatants were collected from Th2 and PTX-Th2 cells 1–3 days after treatment and examined for IL-4 and IL-5 levels. B, Ag-specific proliferation of Th2 and PTX-Th2 cells. The indicated numbers of Th2/PTX-Th2 cells were added to a 96-well plate with APCs and OVAp. Data are means of six replicate wells for each condition. C, Cell surface phenotype of Th2 and PTX-Th2 cells 1–3 days after treatment and examined for IL-4 and IL-5 levels. D, Ag-specific proliferation of Th2 and PTX-Th2 cells. The indicated numbers of Th2/PTX-Th2 cells were added to a 96-well plate with APCs and OVAp. Data are means of six replicate wells for each condition. C, Cell surface phenotype of Th2 and PTX-Th2 cells. Th2 and PTX-Th2 cells were stained with indicated cell surface markers and isotype control Abs at the indicated times and FACS analysis was performed. D, Chemotaxis of Th2 and PTX-Th2 cells in vitro. Migration of Th2 or PTX-Th2 cells was analyzed in response to increasing concentrations of SDF-1α and MDC. Data are presented as the chemotactic index ± SD of one representative experiment performed in quadruplicate. Data are representative of three separate experiments.
surface expression of LFA-1 and VLA-4 on Th2 and PTX-Th2 cells and found no differences between the two groups (Fig. 1C). OVA-specific TCR (KJ) as well as CD4 staining were also unchanged following PTX treatment. However, PTX treatment inhibited chemotaxis of Th2 cells to stromal cell-derived factor 1α/CXCL12 as well as macrophage-derived chemokine MDC/CCL (Fig. 1D). These results indicate that PTX treatment did not alter IL-4 and IL-5 secretion, Ag-induced proliferation, and cell surface expression of LFA-1, VLA-4, and the OVA-specific TCR but did inhibit chemokine-induced chemotaxis of Th2 cells in vitro.

PTX-Th2 cells are unable to traffic into the BAL and lung and induce eosinophilic inflammation and mucus production following adoptive transfer and OVA challenge

To determine whether trafficking of Th2 cells into the lung is chemotactic dependent, we transferred $5 \times 10^6$ Th2 or PTX-Th2 cells by tail vein injection followed by four OVA aerosol challenges (Th2-OVA vs PTX-Th2-OVA). Twenty-four hours after the last challenge, mice were sacrificed and BAL was performed. The total number of cells recovered from the BAL of PTX-Th2-OVA mice were almost 10-fold reduced compared with Th2-OVA mice ($0.32 \times 10^6$ vs $2.26 \times 10^6$ cells) (Fig. 2A). This is similar to mice that did not receive adoptively transferred T cells but were subject to full airway Ag challenge (data not shown). As expected, 60–65% of the cells in the BAL of Th2-OVA mice were eosinophils. In sharp contrast, <5% of the cells were eosinophils in PTX-Th2-OVA mice. The total number of macrophages and lymphocytes were also significantly reduced in the BAL of PTX-Th2-OVA mice compared to those of Th2-OVA mice. Histopathologic examination of lung tissue also revealed a dramatic decrease in overall inflammation and goblet cell mucus production in PTX-Th2-OVA mice compared with Th2-OVA mice (Fig. 2B). Therefore, PTX treatment of Th2 lymphocytes prevented the development of eosinophilic inflammation and goblet cell mucus production in the airways and lungs of mice following adoptive transfer and Ag challenge.

**FIGURE 2.** Attenuated eosinophilic inflammation in BAL of BALB/c mice after transfer of PTX-Th2 cells and aerosol OVA challenge. A, BAL cell counts. Leukocytes were recovered from the BAL after the last aerosol challenge and total and differential counts were performed. Data represent mean number of BAL cells (±SEM; n = 16 for Th2-OVA and n = 19 for PTXTh2-OVA). *, p < 0.001 in Th2-OVA vs PTXTh2-OVA mice. ***, p = 0.007 in Th2-OVA vs PTX Th2-OVA mice. B, H&E-stained Formalin-fixed lung sections indicate characteristic intense inflammatory cell infiltrate comprised of eosinophils and lymphocytes in lung sections of Th2-OVA mice (i) absent in PTXTh2-OVA mice (ii). iii, PAS staining revealed characteristic mucin staining in bronchial epithelium (arrow) of Th2-OVA mice with the presence of subepithelial eosinophils (arrowheads), which are completely absent in PTXTh2-OVA mice (iv). Original magnifications: i and ii, ×100; iii and iv, ×400.

OVA-specific PTX-Th2 cells (KJ+) are markedly diminished in the BAL, lungs, and secondary LNs

To determine whether the lack of eosinophilic inflammation in the BAL of PTX-Th2-OVA mice was due to the inability of the PTX-
Th2 cells to traffic to the sites of Ag challenge, we analyzed the BAL, lung, paratracheal LNs (PLNs), spleen, blood, and inguinal LNs (ILNs) for transferred lymphocytes using flow cytometry. Although we were able to detect KJ* cells in the BAL and lungs of mice following Th2 cell transfer and OVA challenge, the number of KJ* cells recovered from mice that received PTX-Th2 cells were 10-fold reduced in the lung and almost 100-fold in the BAL (Fig. 3F3). PTX-Th2 cells were also detected at significantly lower levels in the PLNs (20-fold) and ILNs (5-fold) with equivalent numbers of transferred cells in the spleen (Fig. 3) and blood (data not shown). These results demonstrate that PTX treatment prevented the migration of Th2 cells to sites of Ag challenge, including the BAL, lungs, as well as secondary LNs, while accumulation of cells in the spleen and blood were not altered.

**Intratracheal instillation of PTX-Th2 and Th2 cells results in the development of eosinophilic inflammation in the BAL of mice following aerosol challenge**

To demonstrate that PTX-Th2 cells were capable of inducing eosinophilic inflammation in vivo, we directly instilled Th2 or PTX-Th2 cells into the trachea of mice, thereby bypassing the need for these cells to traffic into the BAL following aerosol challenge. We speculated that both Th2 and PTX-Th2 cells should be able to secrete cytokines, such as IL-4, IL-5, and IL-13, when stimulated with Ag in vivo and induce allergic inflammation in the BAL. Following intratracheal instillation and OVA aerosol challenges, mice that received PTX-Th2 cells and Th2 cells were both able to develop eosinophilic inflammation (Fig. 4). In fact, mice that received PTX-Th2 cells intratracheally appeared to have more inflammatory cells in the BAL following Ag challenge. Importantly, eosinophils and KJ* lymphocytes were detected in the airways to the same extent in both groups. No eosinophils were detected in BAL of mice following intratracheal instillation of Th2 or PTX-Th2 cells with no OVA aerosol challenge (data not shown), indicating that Th2 and PTX-Th2 cells required aerosol Ag activation. These data clearly demonstrate that PTX-Th2 cells were functional Th2 cells in vivo because when directly instilled into the trachea they were capable of inducing eosinophilic inflammation in the airways following Ag-induced activation.

**Discussion**

The trafficking of Th2 cells to the lung is thought to be a key early step in the pathogenesis of asthma. Although much has been learned about the molecular mechanisms regulating Th1 cell recruitment into tissues (e.g., P-selectin glycoprotein ligand 1, CXCXR3) (8, 9), the molecular mechanisms regulating Th2 cell recruitment remain largely unknown. CCR3, CCR4, and CCR8 are preferentially expressed on Th2 cells in vitro and in vivo and are thought to be the principal chemokine receptors involved in Th2 cell trafficking (2, 10). However, targeted deletion of CCR3, CCR4, and CCR8 did not abrogate Th2 cell-specific trafficking into the lung. Our data demonstrate that trafficking of Ag-specific Th2 cells into the lungs, BAL, and secondary lymphoid tissue is dependent on Gαi-coupled chemokine receptors.

We have previously demonstrated that Th2 lymphocyte recruitment into the lung and airways in allergic pulmonary inflammation is dependent on Stat6 expression in resident cells of the lung. Interestingly, in these mice, the transferred Th2 cells were able to traffic into secondary LNs, suggesting that Th2 trafficking into LNs is not dependent on Stat6-inducible chemokines (6). Taken together, our studies suggest that trafficking of Th2 cells into lung, airways, and LNs is dependent on chemoattractant receptor activation but trafficking of these cells into the lung and LNs is differentially regulated. Trafficking into the lung and airways is dependent on Stat6-inducible chemokines, while trafficking into LNs is independent of Stat6 but dependent on a different subset of chemokines.

Our intratracheal transfer experiments clearly demonstrated that PTX-Th2 cells were functional Th2 cells as they were able to recruit eosinophils into the BAL in vivo. The moderate inflammatory response in comparison to the i.v. transfer of Th2 cells (Figs. 2 and 4) is likely due to our inability to detect intratracheally transferred Th2 cells in any other organs following Ag challenge (data not shown). Although we believe that recruitment of Th2 cells into the lung is a key early step that is essential for chemokine secretion by resident parenchymal cells and subsequent amplification of the inflammatory response, our data also suggest that the trafficking of effector Th2 cells into and out of LNs plays an important role in amplifying allergic inflammation in the airways. It is also interesting to note that dendritic cells have been shown to migrate into draining LNs following intratracheal transfer (11), implying differences in the ability of Th2 cells and dendritic cells to cross the airway epithelial barrier.

In conclusion, our data demonstrate that trafficking of Th2 cells into the lung, airways, and LNs in allergic pulmonary inflammation requires the involvement of functional chemoattractant receptors expressed on Th2 cells and as such represent attractive targets for asthma therapy.

**References**


