The Chemokine Receptor D6 Has Opposing Effects on Allergic Inflammation and Airway Reactivity

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Rationale: The D6 chemokine receptor can bind and scavenge several chemokines, including the T-helper 2 (Th2)-associated chemokines CCL17 and CCL22. Although D6 is constitutively expressed in the lung, its pulmonary function is unknown.

Objectives: This study tested whether D6 regulates pulmonary chemokine levels, inflammation, or airway responsiveness during allergen-induced airway disease.

Methods: D6-deficient and genetically matched C57BL/6 mice were sensitized and challenged with ovalbumin. ELISA and flow cytometry were used to measure levels of cytokines and leukocytes, respectively. Mechanical ventilation was used to measure airway reactivity. **Results:** The ability of D6 to diminish chemokine levels in the lung was chemokine concentration dependent. CCL17 and CCL22 were abundant in the airway, and their levels were attenuated by D6 when they were within a defined concentration range. By contrast, airway concentrations of CCL3, CCL5, and CCL11 were low and unaffected by D6. Allergen-challenged D6-deficient mice had more dendritic cells, T cells, and eosinophils in the lung parenchyma and more eosinophils in the airway than similarly challenged C57BL/6 mice. By contrast, D6-deficient mice had reduced airway responses to methacholine compared with C57BL/6 mice. Thus, D6 has opposing effects on inflammation and airway reactivity.

Conclusions: The ability of D6 to scavenge chemokines in the lung is dependent on chemokine concentration. The absence of D6 increases inflammation, but reduces airway reactivity. These findings suggest that inhibiting D6 function might be a novel means to attenuate airway responses in individuals with allergic asthma.

Keywords: chemokines; lung; D6; allergic; transforming growth factor- β

Allergic asthma is a disease characterized by pulmonary inflammation and reversible airflow obstruction. In allergic asthma, T-helper 2 (Th2) cells recognizing common airborne antigens produce the cytokines IL-4, IL-5, and IL-13, which can account for virtually all of the manifestations of asthma, including eosinophil mobilization and airway remodeling (1). In principle, an effective therapy for asthma would be to inhibit the development of such allergen-specific Th2 cells. However, individuals that present with asthma have already developed Th2 responses to aeroaller-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The D6 chemokine receptor can bind and scavenge several chemokines, including the T-helper 2-associated chemokines CCL17 and CCL22. Although D6 is constitutively expressed in the lung, its function is unknown.

What This Study Adds to the Field

The absence of D6 increases airway inflammation, but reduces airway reactivity. Inhibiting D6 might attenuate airway responses in individuals with asthma.

gens, and immunotherapeutic reversal of this Th2 bias is difficult to achieve. Therefore, it is reasonable to investigate alternative strategies, including the augmentation of natural regulatory mechanisms that attenuate airway hyperresponsiveness.

Leukocyte recruitment to sites of allergic inflammation is controlled largely by a family of chemotactic proteins known as chemokines. The cell specificity of this recruitment is conferred by the array of chemokines produced at inflammatory sites and by the chemokine receptors displayed on leukocyte subsets. Although no chemokine receptor is expressed exclusively on a single cell type, differences in the relative expression of various chemokine receptors likely determine the responsiveness of these cells to individual chemokine ligands. For example, eosinophils display high levels of CCR3 and migrate toward the CCR3 ligand CCL11 (eotaxin) (2–4). Th2 cells display CCR4 and, to a lesser extent, CCR3 and CCR8 (5, 6), whereas Th1 cells display CCR5 and CXCR3. The effectiveness of the CCR4 ligands CCL17 (TARC [thymus and activation-regulated chemokine]) and CCL22 (MDC [macrophage-derived chemokine]) in recruiting Th2 cells (7) suggests that these chemokines might function in Th2-dominated diseases such as asthma. This notion is supported by the findings that the sputum and sera of patients with asthma have elevated concentrations of these chemokines (8–10), and that they are increased in the airway after segmental challenge (11).

In addition to signaling chemokine receptors that direct cells toward chemokine-producing tissues, some chemokine receptors have an opposing function: to bind chemokines and remove them from the inflammatory milieu (12). Such “silent” receptors include D6, which binds ligands of the receptors CCR1, CCR2, CCR3, CCR4, and CCR5 (13) and might dampen inflammation *in vivo* (14). For example, mice lacking D6 have increased levels of multiple chemokines and enhanced inflammation in the skin after topical application of phorbol esters (15, 16). The recent demonstration that D6 effectively scavenges CCL17 and CCL22

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in vitro (17) suggests that this receptor might also impact allergic responses. To test this hypothesis, we studied *D6*-deficient mice in an established model of allergic pulmonary inflammation. We found that, compared with wild-type mice, allergen-challenged *D6*-deficient mice have increased levels of select chemokines and inflammation, but have reduced airway reactivity.

METHODS

Mice

D6-deficient mice (15) were backcrossed for 10 generations onto a C57BL/6J background. Age- and sex-matched control C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the National Institute of Environmental Health Sciences, and experiments carried out in accordance with the standards established by the U.S. Animal Welfare Acts. All mice were between 6 and 12 weeks of age.

Ovalbumin Sensitization and Challenge

Mice were sensitized on Days -13 and -7 by intraperitoneal injections of ovalbumin (Sigma, St. Louis, MO) complexed to alum as previously described (18). Beginning on Day 0, these mice were exposed for 30 minutes to an aerosol of 1% ovalbumin (Sigma) in LPS-free saline (Sigma) generated using an Ultra-Neb99 (DeVilbiss Healthcare, Somerset, PA). For the acute (single-day) challenge protocol, mice were exposed twice on the same day (Day 0) and harvested 48 hours later. For the 7-day challenge, mice were exposed to the aerosol once per day for 7 consecutive days and harvested on the day after the last exposure.

Analysis of Airway Inflammation and Chemokines

Whole lung lavage, cell differential counting, and assays of cytokines by ELISA were performed as described previously (18). ELISA kits were obtained from R&D Systems (Minneapolis, MN).

Flow Cytometric Analysis

Leukocytes were prepared from the lung parenchyma (18), then labeled with monoclonal antibodies against CD11c, CD11b, IA/IE, CD40, CD80, CD86, CD19, CD3, and CD4 labeled with phycoerythrin (PE-H), fluorescein isothiocyanate (FITC) or allophycocyanin (APC) (BD Pharmingen, San Jose, CA). Licensed/mature dendritic cells in the lung mince were identified as major histocompatibility class (MHC) class II (IA/IE)^{high} CD11c^{mod} cells expressing CD40⁺, CD80⁺, or CD86⁺. Leukocytes from mediastinal lymph nodes were prepared similarly, and dendritic cells identified as CD11c⁺ cells. Cells were gated according to size and side scatter to eliminate dead cells and debris from analysis using a FACS Vantage SE instrument (BD BioSciences, San Jose, CA), and the data analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Analysis of Airway Responsiveness

Respiratory mechanics and airway responsiveness to aerosolized methacholine were determined using the FlexiVent mechanical ventilator system (SCIREQ, Inc., Montreal, Canada) as described previously (19). The single-compartment model of the lung was used to assess total respiratory system resistance (R) after delivery of aerosolized methacholine (0 to 25 mg/ml saline) through an ultrasonic nebulizer for 10 seconds without altering the ventilatory pattern, after which the 2-second perturbation was applied consecutively every 30 seconds for 5 minutes. Peak responses during each 5-minute period were determined. Baseline resistance values for each mouse were obtained by applying a 2-second perturbation at a frequency of 2.5 Hz a total of three times, and taking the average of these resistance measurements.

Statistics

Data are expressed as mean $\pm$ SEM. Significant differences between groups were identified by analysis of variance. Individual comparisons between groups were confirmed by Student's *t* test unless otherwise

stated. A two-tailed *p* value of less than 0.05 was considered statistically significant.

RESULTS

Analysis of D6 Scavenging Function in the Lung

We first tested the ability of D6 to scavenge chemokines in the airways of allergen-challenged mice. To induce allergic pulmonary inflammation, we used established models in which mice are first sensitized with ovalbumin and subsequently exposed to an aerosol of this protein on a single day (20), or on 7 consecutive days. Analysis of bronchoalveolar lavage fluid from challenged mice revealed that the CCR4 ligand CCL17 was 10-fold more abundant than any other measured chemokine (Figures 1A and 1B). After the single-day aerosol challenge, CCL17 levels

Figure 1. Analysis of chemokine availability in *D6*-deficient mice. Sensitized mice were exposed to either a single-day (A, C, and E) or a 7-day (B, D, and F) aerosol challenge. Lungs were lavaged and the supernatants assayed for various chemokines. Significant differences in chemokine availability between the two genotypes were seen with CCL17 after the single-day challenge and in CCL22 after the 7-day challenge. Note that the scales for CCL17 and CCL22 are different for the single- and 7-day challenges. Differences between genotypes in which *p* < 0.05 were considered significant.

were approximately threefold higher in *D6*-deficient mice than in C57BL/6 mice ($p < 0.03$). However, after 7 consecutive days of aerosol challenges, when levels of CCL17 had increased dramatically in both *D6*-deficient and C57BL/6 mice, levels of this chemokine were no longer different between the two strains.

The second most abundant chemokine in the lungs of allergen-challenged mice was CCL22, another CCR4 ligand. Mice undergoing the single-day challenge had relatively low levels of CCL22, which were similar in *D6*-deficient and C57BL/6 mice (Figures 1C and 1D). Levels of CCL22 rose markedly in both strains after the more prolonged 7-day challenge, and were significantly higher in *D6*-deficient mice than in C57BL/6 mice ($p < 0.01$). Thus, when present at either very low or very high concentrations, levels of CCL17 and CCL22 were unaffected by D6, whereas when these chemokines were present at moderate levels, they were higher in *D6*-deficient mice than in similarly challenged C57BL/6 mice.

Other chemokines analyzed included the eosinophil chemoattractant CCL11 (eotaxin), as well as CCL3 (macrophage inflammatory protein [MIP]-1 α) and CCL5 (RANTES [regulated upon activation, normal T-cell expressed and secreted]), which are all bound and scavenged by D6 from supernatants of cultured cells (17). Airway levels of CCL11 were low and similar in *D6*-deficient and C57BL/6 mice after the single-day challenge. There was a modest increase in CCL11 in *D6*-deficient mice after 7 days of challenge, but this increase was not statistically significant (Figures 1E and 1F). CCL3 and CCL5 were each present at very low concentrations in the airways with no significant differences between the genotypes after the single-day or 7-day challenge (data not shown).

D6 Attenuates Eosinophil Accumulation in Airways of Allergen-challenged Mice

Eosinophilic inflammation is a hallmark of asthma and of murine models of allergic pulmonary inflammation. The impact of D6 on allergen-provoked eosinophil accumulation in the airways was therefore assessed. As expected, sensitized mice of both *D6*-deficient and wild-type mice challenged on a single day had lower levels of airway eosinophils than mice challenged on 7 consecutive days. However, *D6*-deficient mice had significantly more airway eosinophils than C57BL/6 mice (Figures 2A and 2B), whether the mice were challenged on a single day or on 7 consecutive days. These findings demonstrate that D6 attenuates eosinophil accumulation in allergen-challenged wild-type mice.

Increased Leukocyte Accumulation in the Lungs of Allergic *D6*-deficient Mice

The increased number of eosinophils in airways of *D6*-deficient mice might have resulted either from increased transit of these cells from the blood to the lung interstitium or from there to the airway. To test the former possibility, leukocytes were prepared from the lung interstitium and an antibody directed against CCR3 used to detect eosinophils by flow cytometry (Figures 2C and 2D). These experiments revealed that, as was found for the airway, eosinophil accumulation in the interstitium of *D6*-deficient mice was higher than in similarly challenged C57BL/6 mice (Figures 2E and 2F). Therefore, the increased eosinophils seen in the airways of *D6*-deficient mice likely resulted from enhanced migration of these cells from the blood to the lung interstitium.

Flow cytometry was also used to quantify levels of CD4⁺ T lymphocytes in the lung interstitium (Figures 3A and 3B). Compared with C57BL/6 mice, CD4⁺ T cells were increased in lungs of *D6*-deficient mice after the single-day challenge (Figures 3C and 3D), whereas B cells were increased in *D6*-deficient

Figure 2. Increased eosinophilic accumulation in *D6*-deficient mice. Sensitized mice were exposed to either a single-day challenge (A and E) or a 7-day challenge (B and F). Eosinophils in the airway (A and B) and lung parenchyma (E and F) were identified, the latter by flow cytometric analysis of anti-CCR3 antibody-stained cells (C and D). Shown are compiled results from four separate experiments (A and B), or from one of two representative experiments (E and F).

mice after the 7-day challenge (Figures 3E and 3F). Analyses of licensed dendritic cells expressing CD11c and high levels of MHC II (Figures 4A and 4B) revealed that this cell type was increased approximately 10-fold in both strains after the 7-day challenge compared with the single-day challenge. However, after each of these challenge protocols, *D6*-deficient mice had significantly more dendritic cells than similarly challenged C57BL/6 mice (Figures 4C and 4D). Taken together, these data show that, in wild-type mice, D6 functions to reduce the accumulation of multiple leukocyte subsets in the lung after their sensitization and challenge with allergen.

Increased Leukocyte Accumulation in the Lymph Nodes of Allergic *D6*-deficient Mice

D6 is expressed in the lymphatic endothelium (21), suggesting that trafficking of cells from the lungs to draining lymph nodes might be affected in *D6*-deficient mice. Analyses of lymph nodes

Figure 3. Analysis of lymphocytes in the lung parenchyma of *D6*-deficient mice. Sensitized mice were exposed to either a single-day (C and E) or a 7-day aerosol challenge (D and F). Cells from minced lungs were digested and the recovered cells stained for cell surface markers diagnostic of B cells (CD19) and CD4⁺ T cells (CD3 and CD4). Diagnostic gates for CD4 T cells are shown (A and B). Representative results are presented from one of three experiments, each yielding similar results. FITC = fluorescein isothiocyanate.

harvested from allergen-challenged mice revealed that *D6*-deficient mice had modest but significant increases in dendritic cells after the single-day challenge (Figure 5A). However, after the 7-day challenge, the two strains had similar numbers of dendritic cells (Figure 5B). Similarly, CD4 T cells were also increased in *D6*-deficient mice compared with similarly challenged C57BL/6 mice after the single-day challenge (Figure 5C), but not after the 1-week challenge (Figure 5D). No significant differences between the two strains were seen in numbers of lymph node-derived B cells, regardless of the duration of challenge (Figures 5E and 5F). Taken together with the other data, these observations support the notion that *D6* has wide-ranging effects on leukocyte trafficking to the lung and draining lymph nodes.

Airway Reactivity in Allergic Mice

A fundamental feature of asthma is reversible hyperreactivity of the airway. We therefore studied the impact of *D6* on airway reactivity in ovalbumin-sensitized and -challenged mice. After

Figure 4. Increased dendritic cells in the lung parenchyma of *D6*-deficient mice. Leukocytes were recovered from lungs and stained with isotype control antibodies (A) or antibodies against CD11c and MHC II (B). The gates representing dendritic cells (DCs) and macrophages (mac) are shown. Data are presented for mice challenged on a single day (C) or on seven consecutive days (D). Results shown represent one of three experiments, each yielding similar results. Note the different scales on the y axis for (C) and (D). APC = allophycocyanin.

the single-day challenge, airway responses to methacholine were significantly higher in C57BL/6 mice than in *D6*-deficient mice (Figure 6A), despite the higher levels of inflammation in the latter. By contrast, after the 7-day allergen challenge, no significant differences in airway reactivity were seen between the two genotypes (Figure 6B). The difference in airway reactivity between C57BL/6 and *D6*-deficient mice receiving the single-day challenge might have been due to differential responses to allergen or to basal differences in airway reactivity. We studied airway reactivity in unsensitized and unchallenged C57BL/6 and *D6*-deficient mice. The naive *D6*-deficient mice also had significantly reduced airway responsiveness to methacholine, suggesting that at least some of the difference between allergen-challenged wild-type and *D6*-deficient mice was due to this inherent difference between the two strains.

Acutely Challenged *D6*-deficient Mice Have Increased Airway Levels of Transforming Growth Factor- β 1

To further investigate potential mechanisms underlying the relative decrease in airway responses of acutely challenged *D6*-deficient mice, we analyzed levels of cytokines that have been previously shown to either associate or directly cause airway hyperreactivity. In particular, IL-13 is required for, and can directly cause, airway hyperresponsiveness (22). However, levels of IL-13 were similar in C57BL/6 and *D6*-deficient mice after the single-day challenge and also after the 7-day challenge (Figures 7A and 7B). Levels of the Th2-associated cytokines

Figure 5. Analysis of leukocytes in pulmonary lymph nodes. Sensitized mice were exposed to either a single-day or a 7-day aerosol challenge, and draining lymph nodes from individual animals analyzed separately. Cells were stained for cell surface markers diagnostic of (A and B) licensed dendritic cells, (C and D) CD4⁺ T cells, and (E and F) B cells. Representative results from one of three experiments are shown.

IL-4 and IL-5, as well as levels of the Th1-associated cytokine IFN- γ , were also similar in C57BL/6 and D6-deficient mice (online supplementary data), regardless of whether the mice were challenged on 1 day or on 7 consecutive days. Surprisingly however, levels of both activated and latent transforming growth factor (TGF)- β 1 were significantly higher in D6-deficient mice undergoing the single-day challenge than in similarly challenged C57BL/6 mice, but not in mice undergoing the 7-day allergen challenge (Figure 7). Because TGF- β 1 has been previously reported to diminish airway reactivity (23, 24), a blocking antibody was used to neutralize this cytokine prior to analyzing airway responses in allergen-challenged mice. These experiments did not reveal a significant effect of TGF- β 1 on airway responses of either wild-type or D6-deficient mice (online supplementary data), suggesting that inherent differences in airway responses seen in unchallenged mice are primarily responsible for the

Figure 6. Airway resistance in D6-deficient mice. Mice were analyzed for airway responses to methacholine after being challenged with ovalbumin (ova) on a single day (A; triangles on solid line, C57BL/6 [n = 10]; squares on dotted line, D6^{-/-} [n = 10]) or on 7 consecutive days (B; triangles on solid line, C57BL/6 ova [n = 10]; squares on dotted line, D6^{-/-} ova [n = 10]). Data from naive mice are also presented (C; triangles on solid line, C57BL/6 [n = 19]; squares on dotted line, D6^{-/-} [n = 19]). Significant differences (**p < 0.01 and *p < 0.05) between genotypes are indicated.

strain-specific differences seen in mice undergoing the single-day allergen challenge.

DISCUSSION

The decoy receptor D6 has been previously shown to bind and scavenge multiple chemokines *in vitro*, and to attenuate inflammation in the skin. Although this receptor is constitutively expressed in the lung (13), its impact on pulmonary chemokine levels and inflammation has not been addressed. Based on the high affinity with which D6 binds chemokines associated with allergic responses (17), we hypothesized that this receptor might scavenge chemokines in the lung and thereby attenuate inflammation in airways of allergen-challenged mice. Our findings

Figure 7. Analysis of airway cytokines. Sensitized mice were exposed to either a single-day or a 7-day aerosol challenge. Lungs were lavaged and the cell-free fluid assayed for (A and B) IL-13, (C and D) active transforming growth factor (TGF)-β1, and (E and F) total TGF-β1. Significant differences between the genotypes for * $p < 0.01$ are indicated.

demonstrate that the chemokine-binding activities of D6 *in vitro* do not necessarily predict its impact on chemokine availability *in vivo*. Rather, the ability of D6 to attenuate chemokine levels correlated with the concentration of the chemokine under study. As hypothesized, pulmonary inflammation was increased in *D6*-deficient mice, but they had an unexpected decrease in airway reactivity.

CCL3, CCL5, and CCL11 are all efficiently scavenged by D6 *in vitro*, but their airway levels were not significantly different in allergen-challenged C57BL/6 and *D6*-deficient mice. Only those chemokines whose concentrations in recovered lavage fluid were between 300 and 10,000 pg/ml were significantly higher in *D6*-deficient mice than C57BL/6 mice; chemokines whose concentrations lay outside of this range were not significantly higher in *D6*-deficient versus C57BL/6 mice. CCL17 was within this concentration range in mice undergoing the single-day challenge, but not in mice challenged for 7 days, whereas the converse was true for CCL22. Therefore, the impact of D6 on chemokine levels correlated better with levels of the chemokine under study than with the number of challenges the mice received or their level of pulmonary inflammation. It remains to be seen if this correlation between chemokine concentration and D6 scavenging will also be seen in other models of inflammation.

Analysis of airway eosinophils in the lungs of allergen-challenged mice revealed that these cells were significantly increased in *D6*-deficient mice compared with C57BL/6 mice, even though levels of CCL11 were similar in the two strains. The increased airway eosinophils in the *D6*-deficient mice might have resulted from their increased levels of CCL17, which is a chemoattractant for mouse eosinophils (25). It is also possible that different levels of CCL24 (eotaxin-2) might have contributed to the differences in eosinophils. CCL24 is a potent chemoattractant for mouse eosinophils (25), but it does not have a proline residue at position 2 (26, 27) and may not, therefore, bind D6 efficiently (28). Analysis of CD4⁺ T cells revealed relatively modest increases in *D6*-deficient mice compared with similarly challenged C57BL/6 mice, despite large and significant differences in airway levels of CCL17 and CCL22. This finding is consistent with other studies that have found little or no direct impact of CCR4 on pulmonary inflammation (29, 30), even though CCR4⁺ T cells and their ligands colocalize in human asthma (31). Dendritic cells in the lungs and draining lymph nodes were also increased in *D6*-deficient mice compared with C57BL/6 mice. This finding might also be directly related to increased levels of CCL17 and CCL22 because CCR4 is expressed in dendritic cells (32). However, several other chemokine receptors are also displayed on immature dendritic cells, including CCR1-6 and CXCR4. Thus, possible differences in levels of chemokines binding these receptors might also give rise to the increased number of dendritic cells seen in *D6*-deficient mice.

Our finding that *D6*-deficient mice have reduced airway reactivity compared with C57BL/6 mice was unexpected. IL-13 is a critical molecule for increased airway responses, but levels of this cytokine were similar in C57BL/6 and *D6*-deficient mice, as were levels of IL-4, IL-5, and IFN-γ. Of the cytokines we measured, only TGF-β1 was significantly higher in *D6*-deficient mice than in C57BL/6 mice after the single-day challenge. The previously described ability of TGF-β1 to reduce airway hyperreactivity in mice (23, 33) suggested that the different levels of this cytokine might account for at least part of the increased airway responsiveness seen in allergen-challenged *D6*-deficient mice. However, antibody-mediated neutralization of TGF-β did not significantly affect airway responses in either strain, a result that is consistent with previous findings (34). Therefore, the reduced airway responses in *D6*-deficient mice undergoing the single-day allergen challenge are likely due in large part to the reduced airway responses seen in unchallenged *D6*-deficient mice. It is noteworthy that, in *D6*-deficient mice, prolonged allergen challenge increased airway responses compared with the single-day challenge, whereas this increase was not seen in wild-type mice. It is possible that the higher levels of inflammation in *D6*-deficient mice challenged for 7 days overcome the inherently low airway responses in this strain. Additional studies will be needed to

more fully characterize the relationship between inflammation and airway responses in D6-deficient mice.

The experiments described here extend our knowledge of D6 function *in vivo*. Our studies reveal that D6 is able to reduce the amount of bioavailable CCL17 and CCL22 in the airway, but only when the concentrations of those chemokines were within a defined range. In addition, we have identified a novel function for D6 in airway responsiveness. The high degree of homology between the human and murine D6 genes suggests that D6 might have similar activities in humans. If so, antagonists of D6 function might provide a novel therapeutic avenue to reduce airway hyperreactivity in some clinical settings.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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