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Rapid Up-Regulation of CXC Chemokines in the Airways after Ag-Specific CD4+ T Cell Activation

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Ag-specific activation of CD4+ T cells is known to be causative for the cytokine production associated with lung allergy. Chemokine-induced leukocyte recruitment potentially represents a critical early event in Ag-induced lung inflammation. Whether Ag-specific, lung CD4+ T cell activation is important in lung chemokine production is currently not clear. Using αβ-TCR transgenic BALB/c DO11.10 mice, we investigated the ability of Ag-specific CD4+ T cell activation to induce lung chemokine production and leukocyte recruitment. Within 1 h of exposure of DO11.10 mice to OVA aerosol, lung mRNA and protein for the neutrophil chemokines KC and macrophage inflammatory protein (MIP)-2 were greatly increased. Accordingly, neutrophils in the Airways increased by >50-fold, and KC and MIP-2 proved to be functional because their neutralization significantly reduced airway neutrophilia. CD4+ T cell activation was critical because CD4+ but not CD8+ T cell depletion reduced KC production, which correlated well with the previously observed inhibition of neutrophil influx after CD4+ T cell depletion. In vitro studies confirmed that OVA-induced KC and MIP-2 production was conditional upon the interaction of CD4+ T cells with APCs. A likely secondary mediator was TNF-α, and a probable source of these chemokines in the lung was alveolar macrophages. Thus, Ag-specific CD4+ T cell activation in the lungs leads to rapid up-regulation of neutrophil chemokines and the recruitment of neutrophils to the site of Ag exposure. This may be a key early event in the pathogenesis of Ag-induced lung inflammation. The Journal of Immunology, 2001, 166: 1233–1240.
negative for pathogens. For Ag exposure, the mice were placed in a plexiglass box and exposed to aerosols of PBS alone or PBS with 0.001–5% OVA or 5% BSA (both obtained from Sigma, St. Louis, MO) generated by a Pari Star nebulizer (Pari-Werke, Starnberg, Germany) for a 20-min period. At various time points after Ag exposure, mice were anesthetized (urethane, 1–1.5 g/kg i.p.), and a tracheal cannula was inserted and a BAL was performed with four 0.3-ml aliquots of PBS containing 0.1% BSA and 0.05 mM EDTA. The total number of leukocytes in 20 μl of BAL fluid was determined using a Coulter Counter (Coulter Electronics, Hialeah, FL). Differential leukocyte counts were made by counting 300 cells on stained (Diff-Quik; Dade Diagnostics, Aguada, PR) cytospin preparations by light microscopy using standard morphological criteria.

**Detection of KC and MIP-2 mRNA in the lung**

Lungs were perfused in situ with 100 U/ml heparinized PBS, removed, and cleaned of connective tissue before being snap-frozen in liquid nitrogen. Total RNA was prepared from lungs of experimental and control mice using Trizol Reagent (Life Technologies, Gaithersburg, MD) according to manufacturer instructions. RNA from at least four mice per group was pooled. A 10-μg quantity of total RNA was used in the RNase protection assay. The mCK-5 kit obtained from PharMingen (San Diego, CA) was used to prepare the 35S-ribolabeled RNA probes. Included in the kit were probes for the following mouse chemokines: lymphotactin, RANTES, eotaxin, MIP-1α, MIP-1β, IP-10, monocyte chemotactrant protein-1 (MCP-1), TCA-3, and the housekeeping genes GAPDH and L32. To label, 6 × 10^5 cpmp of probe was added to each sample, and incubations were performed overnight at 37°C. After RNase digestion, samples were run in denaturing polyacrylamide gels. For the Northern blot analysis, samples were run at 65°C in Rapid-Hyb buffer (Amersham International, Amersham, U.K.). Gels were dried and exposed to autoradiographic films or analyzed in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Determination of chemokine protein levels in BAL fluid and lung homogenates**

BAL samples were centrifuged at 1500 rpm for 10 min at 4°C to pellet cells, and supernatants were removed and stored at −80°C. After BAL, lungs were perfused with heparinized saline, removed, and snap frozen in liquid nitrogen. Lungs were homogenized in 3 ml of PBS and centrifuged to clear debris. KC, MIP-2, eotaxin, and MCP-1 levels were determined in BAL fluid supernatants by ELISA as described above. The neutralizing Abs were present for the entire culture period.

**Administration of anti-CD4 and anti-CD8 Abs**

Mice were given an i.p. injection of either rat anti-mouse CD4 (250 μg; GK1.5) or rat anti-mouse CD8 (125 μg; 53-6.7), which have been shown to deplete these cells in vivo (33) or the same amount of the respective isotype control Ab (rat IgG2a or rat IgG2b; all Abs obtained from PharMingen) at 48 and 24 h before 5% OVA aerosol. BAL was performed at 6 h, and the BAL fluid was stored at −80°C for analysis of chemokine levels by ELISA. In these experiments, CD4 and CD8 depletion was confirmed using FACS analysis based on the expression of the murine T cell surface markers, CD4 and CD8 (data not shown). Briefly, peribronchial lymph node were excised into cold DMEM supplemented with 10% FCS. After homogenization and separation over a lymphocyte gradient (Cedarlane Laboratories, Hornby, Ontario, Canada), cells were stained with rat anti-mouse Thy 1.2 in combination with either rat anti-mouse CD4 or rat anti-mouse CD8 (all obtained from PharMingen). Analysis of the lymphocyte population was performed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and confirmed that selective depletion of either CD4+ or CD8+ cells was obtained under the appropriate conditions.

**Administration of KC and MIP-2 neutralizing Abs**

At 30 min before the OVA aerosol, mice were lightly sedated by halothane anesthesia and 50 μg of each of rat anti-mouse KC and rat anti-mouse MIP-2 or isotype controls (rat IgG2a for anti-KC and rat IgG2b for anti-MIP-2; all obtained from R&D Systems) were administered intranasally (i.n.) in a total volume of 100 μl. BAL was performed 6 h after the 5% OVA aerosol, and cells were enumerated as already described.
above baseline, was resolving by 24 h after OVA aerosol exposure (Fig. 2B). Staining of dispersed lung cells with an Ab specific for the transgenic TCR (KJ1-26) and for CD3 (Thy 1.2) and subsequent FACS analysis indicated that the first 6 h of this response to OVA aerosol was not associated with any preferential recruitment of Ag-specific or nonspecific T cells to the lung (data not shown).

**KC and MIP-2 mRNA expression is increased in the lung after OVA aerosol**

Total lung RNA was used to determine the time course of KC and MIP-2 mRNA expression after OVA aerosol. Northern blot analysis was used to detect KC expression, and a RNase protection assay was used to detect MIP-2 expression. KC mRNA was not detected in lungs from naive mice, but was clearly detected in lungs from mice at 1, 3, 6, and 24 h after aerosol exposure. Results are expressed as mean ± SEM of data from four to six mice. Significance (\(p < 0.05\)) was determined in (A) by ANOVA followed by a Dunnett’s modified t test to correct for multiple comparisons and in (B) by Student’s t test and indicates OVA aerosol treatment significantly different from PBS aerosol-exposed mice. KC mRNA expression was determined by Northern blot. Top, KC expression; bottom, loading of the gel. B, Lung MIP-2 expression was determined by RNase protection assay and expression was standardized to expression of the housekeeping gene L32. Significance (\(p < 0.05\)) was determined by ANOVA followed by a Dunnett’s modified t test to correct for multiple comparisons.

**KC and MIP-2 protein are increased in the BAL fluid and lung after OVA aerosol**

Protein levels for various murine chemokines during the first 24 h after 5% OVA aerosol were determined in BAL fluid and lung homogenates by ELISA. Levels of KC and MIP-2 in BAL fluid were greatly increased by OVA aerosol and were maximal by 1 h after aerosol exposure. Although the elevation in MIP-2 expression was much more transient than KC, they both had declined to baseline by 24 h (Fig. 4, A and B). A similar time course for KC and MIP-2 protein production was observed in the lung homogenates prepared from 5% OVA aerosol-challenged mice (Fig. 4, C and D). As an important control, KC levels in BAL fluid were tested 6 h after 5% BSA aerosol. Although slightly elevated above baseline, KC levels after BSA aerosol were much lower than at the corresponding time point in 5% OVA aerosol-exposed mice (2205 ± 53 pg/ml). Differences in eotaxin and MCP-1 expression after OVA aerosol were detected but were much lower (−100-fold) than those seen for KC and MIP-2 and occurred at later time points (Fig. 4, E and F).
**Inhibition of neutrophil influx by neutralizing Abs to KC and MIP-2**

Abs to murine KC and MIP-2 were administered i.n. before OVA aerosol exposure to establish whether these chemokines are functionally involved in neutrophil recruitment in vivo. Administration of anti-KC alone or anti-MIP-2 alone failed to affect neutrophil numbers in BAL at 6 h after 5% OVA aerosol (data not shown). However, the combination of these Abs reduced neutrophil influx seen at 6 h after OVA aerosol by 69%, when compared with the combined isotype control treatment (Fig. 5).

**CD4⁺ T cell depletion reduces OVA aerosol-induced increases in BAL fluid KC levels**

We have previously shown that in vivo depletion of CD4⁺ T cells but not CD8⁺ T cells abrogates the neutrophil influx seen at 6 h in this model (32). To demonstrate that this effect is indeed related to reduced chemokine production, KC levels were measured in BAL fluid after OVA aerosol in mice depleted of CD4⁺ or CD8⁺ T cells. CD4⁺ T cell depletion resulted in a 61% reduction in KC levels detected in BAL fluid measured at 6 h after 5% OVA aerosol (Fig. 6). This correlated with the 66% reduction in neutrophil influx seen after this treatment (32). Differences in MIP-2 protein levels due to CD4⁺ or CD8⁺ T cell depletion could not be determined at the 6-h time point because MIP-2 levels had already returned to near baseline levels (see Fig. 3B).

**KC and MIP-2 production is dependent upon OVA presentation by APCs to CD4⁺ T cells**

The ability of OVA to stimulate chemokine production upon Ag presentation to CD4⁺ T cells was tested in vitro using CD4⁺ T cells isolated from the spleens of BALB/c DO11.10 mice and APCs (irradiated splenocytes) from BALB/c wild-type mice. ELISA analysis of supernatants harvested after 24-h culture revealed that the production of KC and MIP-2 was highest in OVA-stimulated cultures that contained both APCs and CD4⁺ T cells (Fig. 7A). Cultures containing both APCs and CD4⁺ T cells that were stimulated with an irrelevant Ag, HEL, produced much less KC and MIP-2. Both APC and CD4⁺ T cells were required because OVA-stimulated cultures of APCs alone or CD4⁺ T cells alone clearly produced much lower amounts of KC and MIP-2 protein compared with cultures in which both cell populations were present (Fig. 7A). Addition of anti-TNF-α reduced OVA-induced production of KC and MIP-2 by 40 and 55%, respectively (Fig. 7B). Although anti-IFN-γ had some effect on KC production (22% reduction), it failed to reduce MIP-2 production (Fig. 7B).

Anti-CD3-stimulated CD4⁺ T cells produced significant amounts of IFN-γ but failed to produce either KC or MIP-2 (Table 1). BAL performed on naive DO11.10 mice resulted in the recovery of >95% pure population of alveolar macrophages. Upon culture for 24 h, even nonstimulated macrophages produced large amounts of both KC and MIP-2 (data not shown). It appeared that despite care in handling these cells during the process of recovery, alveolar macrophages became activated to produce large amounts of KC and MIP-2.

**Discussion**

Activation of CD4⁺ T cells by Ag is thought to be a key event in the induction of allergic inflammation. Although the production of cytokines is a well recognized outcome of CD4⁺ T cell activation, whether chemokine production is a result of such activation is not clear. Using the BALB/c DO11.10 mouse as an in vivo model of Ag-specific activation of lung CD4⁺ T cells, we have previously shown that OVA aerosol activates lung CD4⁺ T cells and causes airway inflammation (32). At early time points after OVA aerosol, the most striking feature of the inflammatory response seen in DO11.10 mice was an influx of neutrophils into the airway lumen.
In this study, we have further characterized the lung neutrophil influx seen after OVA inhalation. In doing so, we demonstrated that neutrophil chemokines were produced as a result of the interaction of APCs with CD4+ T cells in the context of the specific Ag, OVA. Although the CD4+ T cells themselves were not the source for these chemokines, they were contingent for their production. Such a pathway may be relevant to pathologies of lung allergy that are commonly associated with the infiltration of neutrophils soon after Ag exposure.

Neutrophils and their chemokines in lung inflammation

KC and MIP-2 gene expression and production of their respective proteins was induced very rapidly after OVA aerosol exposure. Consequently, neutrophils were seen in the BAL fluid of DO11.10 mice as soon as 3 h after OVA aerosol exposure. The rapid nature of this response is consistent with the role of these cells as important effector cells in acute inflammation. In the human, IL-8 is thought to be one of the important neutrophil chemoattractants in lung allergy associated with asthma (24, 35). Given the lack of reagents to inhibit the activity of neutrophil chemokines in vivo, this has been difficult to prove definitively. The neutralizing Abs for KC and MIP-2 confirmed that KC and MIP-2 were functional chemoattractants in this mouse model. Even so, the inhibition of neutrophil influx seen with the neutralizing Abs was not complete, suggesting that other chemokines may also be involved. The fact that only the combination of neutralizing Abs for KC and MIP-2 inhibited BAL neutrophil influx suggested that KC and MIP-2 were equally redundant in mediating neutrophil chemoattraction. Consistent with the absence of other leukocytes in the BAL fluid after OVA aerosol, chemokines known to be important in the trafficking of other leukocytes such as eotaxin and MCP-1 (9) were not regulated in a similar time frame or to the same extent as the neutrophil chemokines KC and MIP-2.

The increase in protein for a chemokine seen after an inflammatory insult should be preceded by an increase in expression of its mRNA. Our data suggest that this is the case for KC because increased mRNA for KC was detected by 1 h after OVA aerosol, peaked at 3 h, and was declining by 6 h. Protein levels in both BAL fluid and lung were also elevated by 1 h and remained at this level until at least 6 h after OVA aerosol exposure. However, in the case of MIP-2, there appears to be a less close relationship between mRNA expression and protein production. MIP-2 mRNA expression peaked at 3 h, by which time the protein levels in both the BAL fluid and lung were already declining. Although well below peak levels, MIP-2 protein was still elevated at 6 h after OVA aerosol exposure. The reasons for this aberrant association between MIP-2 protein production and mRNA expression are unclear but there are numerous possibilities. For instance, the inflammatory response might be associated with up-regulation of proteases responsible for the degradation of MIP-2 or else much of the newly synthesized MIP-2 might be truncated to yield a peptide form that could not be detected by the ELISA. N-terminal truncation is commonly seen in chemokines and has been shown to occur for the human neutrophil chemokine granulocyte chemotactic protein-2 (16).

IL-8 and related chemokines are important regulators of neutrophil margination to tissues in many inflammatory settings. It has been shown that the production of neutrophil chemokines correlates with neutrophil influx in LPS (36, 37), particulate dust (38), ozone (39), immune complex (40), bacterial infection (41), and...
chemokine production in allergic responses

lymph node CD4 T cells migrate to the regional lymph nodes of the lung to present Ag to APCs. This interaction led to an impressive neutrophil influx into the lung after Ag challenge. In that study, in vitro work indicated that both Ag-specific and nonspecific activation of mast cells caused the release of ENA-78. They propose that IgE-dependent activation of mast cells may be an important pathway in the production of neutrophil chemoattractants. Up-regulation of neutrophil chemokines seen in the current study is clearly independent of IgE because the exposure to OVA aerosol was a primary event and, in any case, serum total IgE was shown to be at baseline in DO11.10 mice both before and after primary OVA aerosol exposure (32).

Ag-specific activation of CD4+ T cells as a mechanism for chemokine production in allergic responses

In support of a critical role for CD4+ T cells in defining the nature of an inflammatory response in the context of allergy, in vivo depletion of CD4+ T cells but not CD8+ T cells was shown to inhibit the airway neutrophil margination in DO11.10 mice after OVA aerosol exposure (32). Consistent with CD4+ T cells playing a part in neutrophil chemotraction, here we have shown that CD4+ T cell depletion, but not CD8+ T cell depletion, inhibited the increase in neutrophil chemokines seen in the BAL fluid after OVA aerosol. To verify the notion that Ag-specific CD4+ T cell activation could directly lead to production of neutrophil chemokines, we performed in vitro studies using CD4+ T cells and APCs. CD4+ splenocytes isolated from BALB/c DO11.10 mice were stimulated with OVA in the presence of irradiated splenocytes as APCs. This interaction led to an impressive neutrophil chemokine production that was clearly Ag specific because the irrelevant but chicken egg-derived Ag, HEL, failed to produce a similar response. Experiments with anti-CD3-activated CD4+ splenocytes from DO11.10 mice indicated that stimulation through the TCR complex results in significant IFN-γ but not KC or MIP-2 production by CD4+ T cells. Therefore, CD4+ T cells themselves seem unlikely sources for KC and MIP-2. When we cultured alveolar macrophages that were isolated by BAL, even nonstimulated macrophages produced large amounts of KC and MIP-2 (data not shown). This is in line with another study in which cultured alveolar macrophages from humans were shown to produce neutrophil chemokines in the resting state and after LPS stimulation (44). Because alveolar macrophages can also function as APCs, they may be involved in the neutrophil chemokine production seen in our model. Furthermore, the rapid neutrophil chemokine expression and neutrophil influx into the lungs suggested that the lung APCs were able to process OVA and present it to CD4+ T cells very quickly. In such a short time, APCs will not have had time to migrate to the regional lymph nodes of the lung to present Ag to lymph node CD4+ T cells.

Experimental evidence from human and animal models of lung inflammation supports the idea that Ag-specific T cells can play a role in mediating the rapid recruitment of neutrophils to the lung. The model used in this study did not mimic human asthma, as there was no evidence for airway hyperresponsiveness (AHR) in response to either single OVA aerosol challenge or multiple OVA aerosol challenges (32). Using the DO11.10 mouse, our point has been to demonstrate that an early outcome of Ag-specific CD4+ T cell activation in the airways is the up-regulation of neutrophil chemokines that promote the migration of these cells into the lung. These data do not allow us to conclude that neutrophilic inflammation is incapable of inducing AHR, especially because it is likely that there are many factors that might contribute to the development of AHR, including, for instance, the chronicity of the disease. Even so, our findings are particularly interesting in the context of a recent study by Nocker and coworkers using segmental allergen challenge in allergic asthmatics (3). These investigators showed that within 4 h after allergen challenge, IL-8 is produced and neutrophils are recruited to the site of exposure. The lack of an increase in the levels of the mast cell mediator tryptase was suggestive of no mast cell involvement. Thus, CD4+ T cell activation may be a mechanism explaining neutrophil recruitment in response to allergen inhalation.

The ability of CD4+ T cells to respond rapidly to Ag and induce production of chemokines may have significance in occupational asthma in which there is limited evidence for IgE-related immunity but clearly an Ag-specific pathophysiology (48). In these asthmatic patients, although IgE to the sensitizing agent cannot be detected, asthmatic responses can develop quickly upon re-exposure to the Ag (48). In subjects with grain-dust-induced occupational asthma, there was significant IL-8 activity in BAL fluid, and neutrophils predominated in bronchial biopsy specimens (49). Also, in patients with occupational asthma induced by low m.w. compounds, neutrophilic inflammation is dominant over eosinophilic inflammation (50). Therefore, it is conceivable that Ag-specific CD4+ T cell activation resulting in rapid neutrophil chemokine production and

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<th>Isolated CD4+ Splenocytes</th>
<th>Nonstimulated</th>
<th>Anti-CD3 stimulation</th>
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<tbody>
<tr>
<td>IFN-γ</td>
<td>1.8 ± 0.4 ng/ml a</td>
<td>94 ± 10 ng/ml a</td>
</tr>
<tr>
<td>KC</td>
<td>bd</td>
<td>bd</td>
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<td>MIP-2</td>
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a Anti-CD3 stimulation significantly different to nonstimulated culture (p < 0.05).

b BD, Below detection.
neutrophil recruitment may be a pathogenic mechanism in these types of asthma.

In summary, we have shown that Ag-specific activation of CD4+ T cells in the airways of BALB/c DO11.10 mice results in rapid production of functional neutrophil chemokines. This suggests that, similarly to cytokine production, chemokine production may be an important outcome of Ag-specific CD4+ T cell activation. Indeed, CD4+ T cell activation leading to neutrophil chemokine production may explain the neutrophil influx seen commonly in the lungs of allergic asthma after Ag challenge. These data extend the role for CD4+ T cells in allergy from cells that regulate tissue cytokine levels to cells that are also players in the production of chemokines that signal recruitment of effector cells to the site of Ag challenge.

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References


