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Gq Signaling Is Required for Allergen-Induced Pulmonary Eosinophilia

Michael T. Borchers,* Paul J. Justice,† Tracy Ansay,† Valeria Mancino,‡ Michael P. McGarry,* Jeffrey Crosby,† Melvin I. Simon,† Nancy A. Lee,† and James J. Lee2* 

The complexity and magnitude of interactions leading to the selective infiltration of eosinophils in response to inhaled allergens are formidable obstacles to a larger understanding of the pulmonary pathology associated with allergic asthma. This study uses knockout mice to demonstrate a novel function for the heterotrimeric G protein, Gq, in the regulation of pulmonary eosinophil recruitment. In the absence of Gq signaling, eosinophils failed to accumulate in the lungs following allergen challenge. These studies demonstrate that the inhibition of eosinophil accumulation in the airways is attributed to the failure of hemopoietically derived cells to elaborate GM-CSF in the airways. The data suggest that activation of a Gq-coupled receptor(s) on resident leukocytes in the lung elicits expression of GM-CSF, which, in turn, is required for allergen-induced pulmonary eosinophilia, identifying a novel pathway of eosinophil-associated effector functions leading to pulmonary pathology in diseases such as asthma. The Journal of Immunology, 2002, 168: 3543–3549.

Antigen-induced recruitment/activation of proinflammatory leukocytes to the lung as well as activation of resident leukocytes are invariant features of allergic respiratory inflammation. In particular, cytokines, T cells, and T cell-associated secretagogues appear to be contributors to pulmonary pathology resulting in the selective recruitment of eosinophils to the lung (1, 2). This vectorial migration of eosinophils results from both receptor-ligand-mediated activation as well as a series of dynamic interactions between adhesion molecules expressed on eosinophils and the vascular endothelium (3). In addition, the generation of chemokine gradients within the lung appear to be critical to mediate the selective movement of eosinophils (4).

Many receptor-ligand interactions are necessary to elicit eosinophil recruitment (as well as other inflammatory responses). In addition, there are a variety of intracellular signaling events required to transduce these receptor-mediated interactions. Heptahedral transmembrane receptors coupled to G proteins (the largest family of cell surface proteins in the human genome (5)) are capable of responding to many forms of stimuli, including as is the case for proinflammatory leukocytes, gradients of chemotactic ligands leading to activation and tissue-specific recruitment (6). The function of these cell surface receptors is controlled by 16 GTP binding G proteins that can be classified into four subfamilies of G proteins, Goq, Gai, Goq, and G12. G protein signal transduction mediates cellular responses by regulating second messenger activities, including phospholipases, adenyl cyclases, phosphodiesterases, and ion channels (7). Thus, the combinatorial interaction of multiple receptors generates a large number of permutated signaling pathways, leading to unique stimulus-response reactions.

The Gq family, includes four subtypes Gq11, Gq14, and Gq15/16. Of particular interest is one member of this family, Gq, that is expressed in many of the leukocytes/tissues involved in allergic inflammatory reactions, such as the thymus and spleen (all hemoipoietic cell types examined) (8), lung epithelium (9), and endothelial cells (10). In addition, it is noteworthy that Gq-coupled receptors have been linked to the induction of the NFAT family of transcription factors (11), potential regulators of early immune response cytokine expression (e.g., IL-4) involved in T cell differentiation/activation and the development allergic inflammation (12, 13). Moreover, Gq protein expression is increased in guinea pig lungs following Ag challenge (14); however, the potential function(s) of Gq in allergen-induced pulmonary inflammation have not been investigated.

In this study mice deficient in the Gqα subunit were used to investigate its potential role(s) in allergen-induced recruitment of eosinophils to the lung. The data demonstrate a required role for Gq signaling in the development of allergen-induced airway eosinophilia. This effect appears to be mediated by failure of the knockout mice to elaborate pulmonary levels of GM-CSF in response to allergen. The Gq signaling defect is limited to a marrow-derived cell(s), but was independent of T cell responsiveness to Ag and eosinophil chemotaxis.

Materials and Methods

Mice

Gq-deficient mice were generated by homologous recombination as previously described (15). Compound Gq−/−/IL-5 transgenic mice were obtained by crossing Gq−/− animals with mice constitutively expressing IL-5 from peripheral T cells (16). All procedures were conducted on specific pathogen-free mice 8–12 wk of age maintained in ventilated microisolate cages housed in an American Association for Accreditation of Laboratory Animal Care-accredited animal facility. Protocols and studies involving animals were conducted in accordance with National Institutes of Health and Mayo Clinic Foundation guidelines.
Assessment of allergic inflammation

The OVA model of allergic pulmonary inflammation has been previously described (17). Briefly, mice (20–30 g) were sensitized by an i.p. injection (100 μl) of 20 μg chicken OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 2 mg Injekt Alum (Al(OH)3/Mg(OH)2; Pierce, Rockford, IL) on days 0 and 14. Mice were subsequently challenged with an aerosol of 1% OVA in saline or saline alone on days 24, 25, and 26. Eosinophil accumulation was assessed on days 27, 28, and 29 by enumerating bronchoalveolar lavage (BAL)3 leukocytes as previously described (17). Cell-free BAL fluids and serum were flash-frozen in liquid nitrogen and stored at −80°C before cytokine level determination by ELISA. Assessments of blood leukocytes were performed on day 28 on both peripheral blood (viz, the tail vasculature) and femoral bone marrow as previously described (16).

Immunohistochemistry and assessment of peribronchial eosinophils

Immunohistochemistry was performed using a rabbit polyclonal Ab against mouse major basic protein (MBP). MBP Ab (1/100 dilution) was allowed to adhere to plates overnight at 4°C before cytokine level determination by ELISA. Mouse IL-4, IL-5, IFN-γ, and GM-CSF ELISA kits from R&D Systems (Minneapolis, MN) were used according to the manufacturer’s protocol. The limits of detection for each assay were: IFN-γ, <30 pg/ml; IL-4, <10 pg/ml; IL-5, <10 pg/ml; and GM-CSF, <5 pg/ml.

Instillation of GM-CSF into OVA-challenged mice

Immediately following each OVA challenge (i.e., days 24, 25, and 26), 1 ng recombinant mouse GM-CSF (R&D Systems) in PBS/0.1% BSA or vehicle alone was instilled intranasally into lightly anesthetized mice.

Statistical analysis

Data presented are the mean ± SE. Statistical analysis was performed on parametric data using t tests with differences between means considered significant when p < 0.05.

Results

Allergen-induced airway eosinophilia is significantly lower in Gq−/− mice

Airway and peribronchial eosinophil accumulations are hallmark features of allergic pulmonary models in the mouse, with peak eosinophil accumulation in the lung typically occurring 24–48 h following the last allergen challenge. In Gq−/− mice, however, the numbers of eosinophils in the BAL (Fig. 1A) following OVA challenge was significantly lower compared with wild-type mice. The inhibition of BAL eosinophil accumulation was observed at all time points examined over 72 h, precluding the likelihood of a transient early or delayed increase in Gq−/− mice. This effect on pulmonary eosinophil accumulation is reflective of the total number of cells recovered in the BAL of Gq−/− mice, which was consistently lower than that in wild-type mice (30–40% reduction). Significant numbers of eosinophils were not recovered from the lungs of saline-challenged mice of either genotype (data not shown).

Histological examination of the lungs from OVA-challenged mice demonstrated a similar lack of peribronchial eosinophil accumulation in Gq−/− mice compared with wild-type animals (Fig. 1). However, this reduction in airway eosinophils was not associated with decreased levels of circulating Th2 cytokines. Serum IL-4 and IL-5 levels following Ag challenge were significantly different between wild-type and Gq−/− mice (IL-4, 64.7 ± 12.6 and 68.7 ± 18.2 pg/ml, respectively; IL-5, 82.8 ± 27.5 and 99 ± 23.1, respectively). Serum cytokine levels in saline-challenged control mice of either genotype were below the limit of detection.

The cell number and composition of bone marrow and peripheral blood are unaffected in Gq−/− mice

The expression of Gq in a wide array of leukocytes (8) suggested that the loss of this signaling pathway would lead to perturbations in hemopoietic compartments and thus account for the absence of a pulmonary eosinophilia in Gq−/− mice. However, no significant differences in the percentages of granulocytes, lymphocytes, or mononuclear cells were observed in Gq−/− mice relative to wild-type controls following OVA challenge (Fig. 2, A and B). Moreover, the total number of cells recovered from the bone marrow or peripheral blood was not different among mice of either genotype (data not shown).

Gq signaling in a marrow-derived cell type(s) is required for the development of OVA-induced pulmonary eosinophilia

The expression of Gq in hemopoietically derived cells as well as structural cells of the lung (8) necessitated an initial assessment of contributing Gq signaling events from either compartment. Bone

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3 Abbreviations used in this paper: BAL, bronchoalveolar lavage; MBP, major basic protein.
marrow engraftment studies were preformed, adoptively transferring either wild-type or Gq<sup>-/-</sup> marrow into wild-type recipients. Engrafted mice were subsequently sensitized/challenged with OVA to determine whether the reduction in airway eosinophilia was primarily a consequence of a marrow-derived cell or defects associated with one or more nonhemopoietic lineages. The response to OVA challenge in wild-type mice receiving wild-type marrow was indistinguishable from that in nonirradiated wild-type animals. However, adoptive engraftment of Gq<sup>-/-</sup> marrow into wild-type mice resulted in a significant decrease in airway eosinophil accumulation following OVA challenge (Fig. 3). These data demonstrate that perturbations of intracellular signaling in one or more lympho-hemopoietic cell types are responsible for the observed decrease in airway eosinophilia.

**FIGURE 1.** The recruitment of eosinophils to the airways of OVA-sensitized/challenged Gq<sup>-/-</sup> mice is significantly reduced compared with that in wild-type animals. A, BAL-derived eosinophils were assessed as a function of time after OVA challenge (control groups received a saline-only challenge) in wild-type vs Gq<sup>-/-</sup> mice. *, Significantly different (<i>p</i> < 0.05) from wild-type mice. Eosinophils comprise <1% of leukocytes in the airways of saline-challenged mice of either genotype. Values presented are the mean ± SE (n = 8–10 mice/group). B–E, Trafficking of eosinophils to the peribronchial regions of wild-type and Gq<sup>-/-</sup> lungs was assessed by immunocytochemistry using a rabbit polyclonal Ab specific for mouse MBP. Saline control groups: B, wild-type; D, Gq<sup>-/-</sup>. OVA-challenged groups: C, wild-type; E, Gq<sup>-/-</sup>. Photomicrographs are from representative sections taken from five mice per group. F, Quantification of peribronchial eosinophil accumulation in wild-type and Gq<sup>-/-</sup> mice. No differences in the number or specific location of eosinophils recruited to the lung were observed between similarly treated mice of either genotype. Scale bar = 100 μm.

**FIGURE 2.** Levels of marrow-derived and circulating leukocytes are unaffected in Gq<sup>-/-</sup> mice. No significant differences (<i>p</i> < 0.05) was observed in the composition of femoral marrow-derived (A) or peripheral blood (B; viz, the tail vasculature) leukocytes from wild-type vs Gq<sup>-/-</sup> mice following saline or OVA challenge (day 28). Values presented are the mean ± SE (n = 4–5 mice/group).

Response to OVA challenge in wild-type mice receiving wild-type marrow was indistinguishable from that in nonirradiated wild-type animals. However, adoptive engraftment of Gq<sup>-/-</sup> marrow into wild-type mice resulted in a significant decrease in airway eosinophil accumulation following OVA challenge (Fig. 3). These data demonstrate that perturbations of intracellular signaling in one or more lympho-hemopoietic cell types are responsible for the observed decrease in airway eosinophilia.

**Eosinophils deficient of Gq migrate in response to chemoattractants**

Eosinophil chemotaxis following allergen challenge is controlled by concurrent G protein-coupled receptor-ligand interactions (3), suggesting that potential signaling deficiencies lie within the eosinophil itself. In vitro Transwell migration assays were used to assess potential cell autonomous effects of Gq signaling on eosinophil migration. No differences were observed in eotaxin-1-mediated chemotaxis of wild-type vs Gq<sup>-/-</sup> eosinophils (Fig. 4A). Furthermore, the migration of Gq<sup>-/-</sup> deficient eosinophils was unaffected (relative to wild-type) in response to other chemoattractants shown to bind, and signal through, distinct receptors on these cells (e.g., platelet-activating factor and complement factor C5a (our unpublished observations); Fig. 4A). Collectively, these data show that Gq signaling is an unlikely causative event(s) leading to OVA-induced pulmonary eosinophil recruitment.
Systemic T cell responses occur in OVA-treated \( G_q^{-/-} \) mice

T cell activity was assessed in \( G_q^{-/-} \) mice to determine whether the loss of allergen-induced pulmonary eosinophilia was a consequence of \( G_q \)-dependent effects on T cell function. No differences were observed in the ability of splenocytes isolated from wild-type or \( G_q^{-/-} \) mice to elaborate IL-4 and IFN-\( \gamma \) in response to the mitogen Con A or nonspecific T cell activation, viz, the cross-linking of TCRs (i.e., anti-CD3; Fig. 4, B–E). In addition, Ag recall assays demonstrated that lymphocytes and APC from \( G_q^{-/-} \) mice were able to elicit Th2 cytokine production in vitro upon exposure to OVA. Splenocytes from \( G_q^{-/-} \) mice generate equivalent amounts of IL-4 in response to Ag stimulation (Fig. 4F), showing that \( G_q \) is not required to generate a memory response toward a particular Ag.

Pulmonary production of GM-CSF, but not Th2 cytokines, is dependent on \( G_q \) signaling

Local immune responses (i.e., BAL cytokine levels) potentially leading to, and/or augmenting, eosinophil accumulation in the lung following OVA challenge were assessed in \( G_q^{-/-} \) mice. Twenty-four hours following the first (day 24) OVA aerosol challenge (i.e., the kinetic maxima of cytokine levels in this protocol (20)) the production of lymphocyte-derived Th2 cytokines (e.g., IL-4 and IL-5) was unaffected in \( G_q^{-/-} \) mice (Fig. 5). However, local production of GM-CSF was significantly reduced as a consequence of the \( G_q \) deficiency. These data show that the pulmonary level of GM-CSF increases in OVA-treated wild-type mice from an undetectable level (before OVA challenge) to \( \sim 40 \) pg/ml. In contrast, OVA treatment of \( G_q^{-/-} \) mice led only to an nominal increase in GM-CSF levels (\( \sim 5 \) pg/ml), representing an 88% reduction relative to OVA-treated wild-type animals.

The loss of local GM-CSF production was demonstrated as being fundamental to the inhibition of eosinophil accumulation in \( G_q^{-/-} \) mice by instillation of recombinant cytokine. Intranasal administration of 1 mg mouse rGM-CSF into \( G_q^{-/-} \) mice immediately following each OVA challenge (i.e., days 24, 25, and 26), recovered the ability to develop airway eosinophil accumulation; this eosinophilia was similar to levels observed in wild-type mice.
The effect of mouse rGM-CSF instillation was specific to OVA-treated $G_q^{-/-}$ mice as the introduction of rmGM-CSF into the airways of saline-challenged mice of either genotype or of OVA-treated wild-type animals had no effect on eosinophil levels.

**Discussion**

$G_q$ expression (and presumably $G_q$ signaling) is widely distributed in the mouse, occurring in nearly every tissue examined (8). Surprisingly, despite this tissue/cell distribution, effects of $G_q$ deficiency on allergen-induced recruitment of eosinophils to the lung were limited to a marrow-derived cell(s). However, the identification of a responsible cell-type linking $G_q$ signaling and the loss of allergen-induced pulmonary eosinophilia remains problematic.

Eosinophils themselves express several receptors that are coupled to heterotrimeric G proteins, many of which are involved in the migration and activation of these cells in response to inflammatory mediators. For example, G protein-coupled receptors involved in eosinophil migration include receptors responsible for the binding and signaling of a diverse group of mediators, including chemokines (21, 22), leukotriene B4 (23), platelet-activating factor (23), complement factor 5a (23), PGD2 (24), and neuropeptides (25). Interestingly, the majority of the responses reported for these receptors have been demonstrated to be pertussis toxin-sensitive, indicating the involvement of the Gi or Go family of heterotrimeric G proteins. The ability of $G_q^{-/-}$ eosinophils to migrate with equal potency as wild-type eosinophils in response to several ligands with eosinophil agonist activities (i.e., eotaxin-1, C5a, and platelet-activating factor) supports this apparent independence of $G_q$ signaling. $G_q$ expression has been detected in many mouse tissues and leukocytes, including macrophage, T cells, and B cells (8). However, the expression of $G_q$ in mouse (or human) eosinophils has not been previously examined. Significantly, we were unable to detect $G_q$ expression in mouse eosinophils by Western blot or sequencing of RT-PCR products using degenerate primers for G9 subunits (data not shown). This would suggest that the absence of $G_q$ signaling in eosinophils does not account for the failure of $G_q^{-/-}$ mice to develop pulmonary eosinophilia.

Allergic pulmonary inflammation, including the specific accumulation of eosinophils in the lung, is a process regulated by T cells (26). In particular, the expression of Th1/Th2 cytokines have been implicated as a root cause of eosinophil accumulation, eliciting both effects directly on eosinophil proliferation and/or survival (e.g., IL-5 (27) and IFN-γ (28)) as well as indirect mechanisms enhancing pulmonary eosinophil recruitment (e.g., IL-4/IL-13 (29–31)). Moreover, in vitro studies have implicated $G_q$ signaling in the induction of NFAT (11), a family of transcription factors potentially involved in the regulation of cytokine expression following stimulation of the TCR complex (32). However, the loss of allergen-induced pulmonary eosinophilia in $G_q^{-/-}$ mice is probably not a consequence of an impaired memory response or
the ability to generate a Th2 response. T cells from Gq−/− mice were able to produce IL-4 and IFN-γ in response to either Con A stimulation or TCR activation. In addition, T cells isolated from OVA-sensitized knockout mice were also able to initiate a Th2-specific immune response following OVA restimulation in vitro. Interestingly, IFN-γ was not detected above baseline values (data not shown) following in vitro OVA restimulation, indicating that the lack of an airway eosinophilia is also not the result of a Th1-skewed cytokine balance that would inhibit the accumulation of eosinophils (28).

The resolution of this quandary regarding a cell autonomous defect associated with the Gq-deficiency probably resides in the unique loss of GM-CSF production in the lungs of Gq−/− mice. The link between local GM-CSF production and allergen-induced eosinophil accumulation in the lung is multifaceted, including increased effectiveness of Ag presentation to T cells (33–35), increased eosinophil survival (36, 37), and enhanced eosinophil migration (38, 39). Local production of GM-CSF in the lungs of asthma patients is primarily confined to macrophages (40), T cells (41, 42), eosinophils (43), and epithelial cells (44). Similar increases in pulmonary GM-CSF levels have also been demonstrated in the lungs of mice following allergen sensitization/challenge (20). The allergen challenge studies of mice following adoptive engraftment of wild-type vs Gq−/− marrow eliminate epithelial cells as a prominent contributor of pulmonary GM-CSF.

The identity of the cellular source of GM-CSF (and presumably the target cell of the Gq deficiency) is unresolved, but may include T cells and/or alveolar macrophages. Macrophages, in particular, are a prodigious source of GM-CSF (45); they are a predominant resident cells in the lung (i.e., alveolar macrophages are present before allergen provocation), and evidence in the literature suggests potential Gq-dependent pathways in the macrophage that may lead to the elaboration of GM-CSF. For example, endothelins are small peptides released into the lung during the initial phase of inflammation and further elucidates the mechanisms mediating the eosinophil accumulation that occurs in diseases such as asthma.

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References


INHIBITION OF EOSINOPHIL RECRUITMENT IN Gq−/− MICE

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