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$G\alpha_{i2}$-mediated signaling events in the endothelium are involved in controlling leukocyte extravasation

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The trafficking of leukocytes from the blood to sites of inflammation is the cumulative result of receptor-ligand-mediated signaling events associated with the leukocytes themselves as well as with the underlying vascular endothelium. Our data show that $G\alpha_i$ signaling pathways in the vascular endothelium regulate a critical step required for leukocyte diapedesis. *In vivo* studies using knockout mice demonstrated that a signaling event in a non-lymphohematopoietic compartment of the lung prevented the recruitment of proinflammatory leukocytes. Intravital microscopy showed that blockade was at the capillary endothelial surface and *ex vivo* studies of leukocyte trafficking demonstrated that a $G\alpha_i$-signaling event in endothelial cells was required for transmigration. Collectively, these data suggest that specific $G\alpha_{i2}$-mediated signaling between endothelial cells and leukocytes is required for the extravasation of leukocytes and for tissue-specific accumulation.

G proteins | inflammation | knockout mice | leukocyte trafficking | pulmonary models

The tissue-specific recruitment of polymorphonucleated granulocytes (i.e., neutrophils, basophils, and eosinophils) and lymphocytes has been particularly well studied, including the mechanisms mediating leukocyte tethering, rolling, adhesion, and eventual transmigration from the circulation (1). These studies have suggested that receptor–ligand interactions coupled to $G\alpha_i$-containing heterotrimeric G proteins in leukocytes are particularly important for the vectorial movement of leukocytes to tissues in response to chemokine gradients (2). Thus, these receptors represent potential drug targets for therapeutic approaches directed against inflammatory diseases (3).

The activation of $G\alpha_i$-coupled receptors leads to the dissociation of the heterotrimeric G protein and intracellular signaling events mediated by the release of the $G\alpha_i$ subunit ($G\alpha_i$) bound to GTP and the free $G\beta\gamma$ dimer (4). The $G\alpha_i$ family includes genes encoding the subunits $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, and $G\alpha_z$ (5). The activities of $G\alpha_i$ family members, with the exception of $G\alpha_z$, are distinguishable from other $G\alpha$ subunits by their susceptibility to pertussis toxin (PTX). The $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ subunits are expressed in many leukocytes and tissues involved in allergic inflammation such as granulocytes (6), lymphocytes (7), airway smooth muscle (8), airway epithelium (8), and endothelial cells (9). Leukocytes that express these $G\alpha_i$ family members appear to express all three $G\alpha_i$ subunits simultaneously. However, studies assessing expression and function have suggested that heterotrimeric complexes containing $G\alpha_{i2}$ and $G\alpha_{i3}$ are abundant in leucocytes (2). Moreover, the importance of $G\alpha_i$-mediated signaling events in leukocyte recruitment/accumulation has been highlighted by using mouse models capitalizing on the inhibitory character of PTX (6). However, the specific mechanisms and, more importantly, the relevant cell types involved in facilitating leukocyte accumulation remain largely unknown.

Results and Discussion

$G\alpha_{i2}$-Signaling Pathways in a Resident Cell of the Lung Are Required for the Accumulation of Eosinophils After Allergen Provocation. The recruitment and accumulation of eosinophils in the airway lumen and lung tissue after allergen provocation is a defining feature found in both asthma patients (10) and animal models of allergic respiratory inflammation (11). $G\alpha_i$-coupled CCR3 receptor–ligand interactions promoting chemotaxis are primarily responsible for the allergen-induced accumulation of pulmonary eosinophils (12). $G\alpha_{i2}$ and $G\alpha_{i3}$ transcripts dominate the mRNAs encoding the PTX-sensitive $G\alpha_i$ subunits of mouse peripheral blood eosinophils (supporting information (SI) Fig. 5). We therefore examined mice deficient in either $G\alpha_{i2}$ or $G\alpha_{i3}$ for airway eosinophilia during asthma induced by sensitization and aerosol challenge with ovalbumin (OVA). The accumulation of eosinophils in the airway lumen of $G\alpha_{i2}{}^{-/-}$ mice was significantly reduced relative to wild-type animals (Fig. 1*A*), but induced eosinophilia in $G\alpha_{i3}{}^{-/-}$ was unaffected (Fig. 1*B*). A similar reduction in eosinophil accumulation was seen in the peribronchial areas of the lungs from $G\alpha_{i2}{}^{-/-}$mice but not $G\alpha_{i3}{}^{-/-}$ mice relative to wild-type animals (SI Figs. 6 and 7). These data indicate that $G\alpha_{i2}$-dependent signaling pathways are required for eosinophil entry into tissues from the circulation.

In vitro chemotaxis assays demonstrated that the loss of $G\alpha_{i2}$ signaling in eosinophils did not prevent $G\alpha_i$-coupled receptor-mediated chemotaxis (e.g., CCR3-mediated responses to eotaxin-1/-2; ref. 12); instead, the loss of $G\alpha_{i2}$ may have even enhanced the ability of the granulocytes to respond to chemoattractant (Fig. 1*C*). Furthermore, these studies showed that the eotaxin-CCR3-mediated chemotactic response of $G\alpha_{i2}$-deficient eosinophils was abolished by PTX (Fig. 1*D*), demonstrating that the signal transduction pathways mediating chemotaxis used the

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The authors declare no conflict of interest.

Abbreviations: PTX, pertussis toxin; OVA, ovalbumin; VCAM, vascular cell adhesion molecule; BAL, bronchoalveolar lavage; PBS, phosphate-buffered saline.

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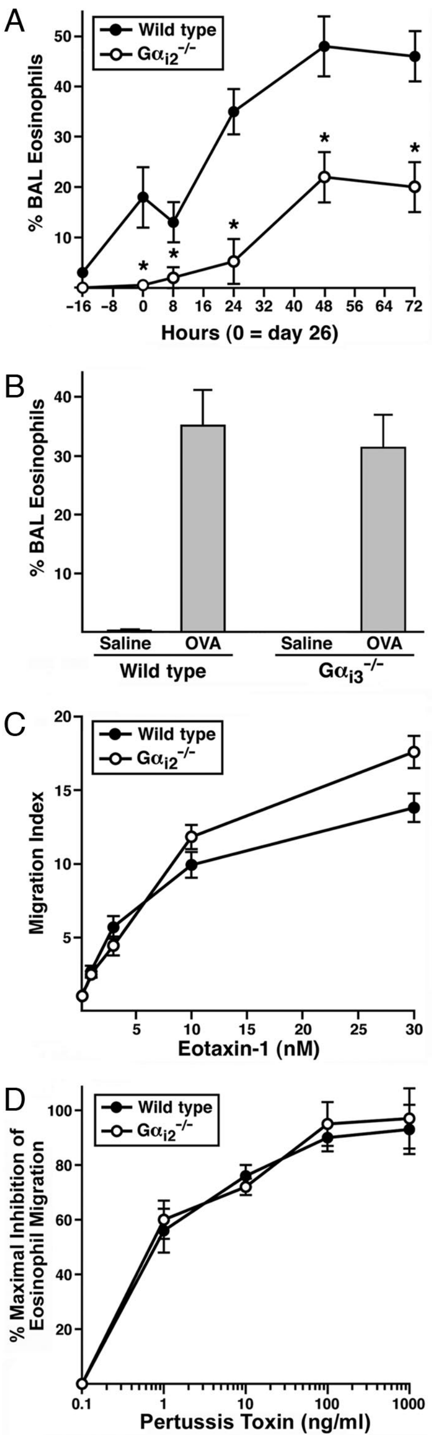

Fig. 1. Allergen-induced eosinophil accumulation uniquely relies on $G\alpha_{i2}$ signaling mechanisms that are independent of cell autonomous events in eosinophils. OVA-induced eosinophil accumulation in the airway lumen of $G\alpha_{i2}^{-/-}$ (*A*), but not $G\alpha_{i3}^{-/-}$ (*B*), mice was significantly lower (*, $P < 0.05$) relative to wild-type animals ($n = 8$ mice per group). (C) Eosinophil transwell chemotaxis assays demonstrated that in the absence of $G\alpha_{i2}$ signaling events, *in vitro* eosinophil migration to recombinant mouse eotaxin-1 was the same, if not nominally higher, relative to wild type. (*D*) PTX pretreatment of eosinophils before the transwell chemotaxis assay showed that the blockade of all $G\alpha_i$-signaling events abolished eotaxin-1-induced chemotaxis, thus demonstrating that the CCR3 receptor-mediated eosinophil chemotaxis occurring in the absence of $G\alpha_{i2}$ results exclusively from signaling events using the remaining PTX-sensitive $G\alpha$ subunits, $G\alpha_{i1}$ and/or $G\alpha_{i3}$.

remaining $G\alpha_i$ family members. These data support the hypothesis that $G\alpha_i$ signaling events modulate eosinophil responses to available chemoattractants *in vitro*. However, the loss of allergen-induced pulmonary eosinophilia in $G\alpha_{i2}$ knockout mice is unlikely to be a consequence of an eosinophil-specific $G\alpha_{i2}$ deficiency but instead might depend on $G\alpha_{i2}$ in other cells or on other signals.

Adoptive transfer of wild-type vs. $G\alpha_{i2}^{-/-}$ eosinophils into OVA-treated wild-type recipient mice was performed as an *in vivo* approach to confirm our *in vitro* observation that the loss of $G\alpha_{i2}$ in eosinophils appeared to enhance chemotactic responses and therefore was not directly responsible for the lack of eosinophil accumulation observed in the lungs of OVA-treated $G\alpha_{i2}^{-/-}$ mice. IL-5 transgenic mice (NJ.1638; ref. 13) or compound $G\alpha_{i2}$-deficient IL-5 transgenic animals ($G\alpha_{i2}^{-/-}$/NJ.1638) were used to isolate pure (>98.5%) populations of wild-type and $G\alpha_{i2}^{-/-}$ peripheral blood eosinophils, respectively. Adoptive transfer was achieved by the repeated i.p. instillation of eosinophils into sensitized wild-type mice during the challenge phase of the OVA protocol. In some studies, the eosinophils were labeled *ex vivo* with the fluorescent tag carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE; Molecular Probes Inc., Eugene, OR) to confirm the identity of the eosinophils in the lung as being derived from those adoptively transferred (SI Fig. 8). Assessment of airway cell populations after adoptive transfer of wild-type eosinophils demonstrated that their increased availability in circulation resulted in a greater number of airway eosinophils (Fig. 2*A*). However, the adoptive transfer of the same number of $G\alpha_{i2}^{-/-}$ eosinophils into OVA-treated wild-type animals resulted in an elevated OVA-induced airway eosinophilia that was even greater than the increase observed after transfer of wild-type eosinophils (Fig. 2*A*). This pattern also extended to lung tissue where transfer of $G\alpha_{i2}^{-/-}$ eosinophils resulted in higher levels of accumulation compared with transfer of wild-type eosinophils (Fig. 2*A* and SI Fig. 9). These transfer studies collectively confirmed our *in vitro* transwell chemotaxis results and showed that $G\alpha_{i2}^{-/-}$ eosinophils are capable of responding to chemotactic gradients and, if anything, may have a greater inherent ability for migration relative to wild-type eosinophils. Interestingly, the reciprocal transfer of wild-type eosinophils into OVA-treated $G\alpha_{i2}^{-/-}$ mice did not result in a pulmonary eosinophilia. Thus, allergen-induced recruitment of eosinophils in this model was absolutely dependent on the presence of a $G\alpha_{i2}$ signaling pathway in the recipient mice, regardless of the $G\alpha_{i2}^{-/-}$ status of the eosinophils (Fig. 2*A*).

The widespread expression of $G\alpha_{i2}$ among all leukocytes, including cells necessary for acquired immune responses (e.g., dendritic cells, B cells, and T lymphocytes; ref .4), suggested the possibility that the observed decrease in eosinophil accumulation in the lungs of OVA-treated $G\alpha_{i2}^{-/-}$ mice was simply a consequence of signaling defects limiting immune responses with a consequent loss of eosinophilia. To test this hypothesis, we examined asthma-induced eosinophilia in bone marrow reconstituted mice in which all hematopoietic, but not the other host cell, lacked $G\alpha_{i2}$. OVA sensitization and challenge of mice reconstituted with wild-type cells revealed bronchoalveolar lavage (BAL) eosinophilia comparable to normal mice. In contrast the eosinophilic response in mice reconstituted with $G\alpha_{i2}^{-/-}$ bone marrow was nearly 3-fold greater. Thus the requirement for $G\alpha_{i2}$ in asthma-induced eosinophilia is not dependent on lymphohematopoietic cells. In accord with this finding, the immune response itself during OVA-induced asthma was not reduced in $G\alpha_{i2}^{-/-}$ mice, as assessed by production of IL-4, IL-5, or IFN-γ (Fig. 2*C*). Moreover, OVA treatment of irradiated wild-type recipients after engraftment of wild-type vs. $G\alpha_{i2}^{-/-}$ bone marrow also displayed equivalent BAL cytokine levels, demonstrating that the $G\alpha_{i2}$-dependent differences in pulmonary eosinophil accumulation were not the result of compromised OVA-induced immune responses (Fig. 2*C*). Attempts to do the reciprocal

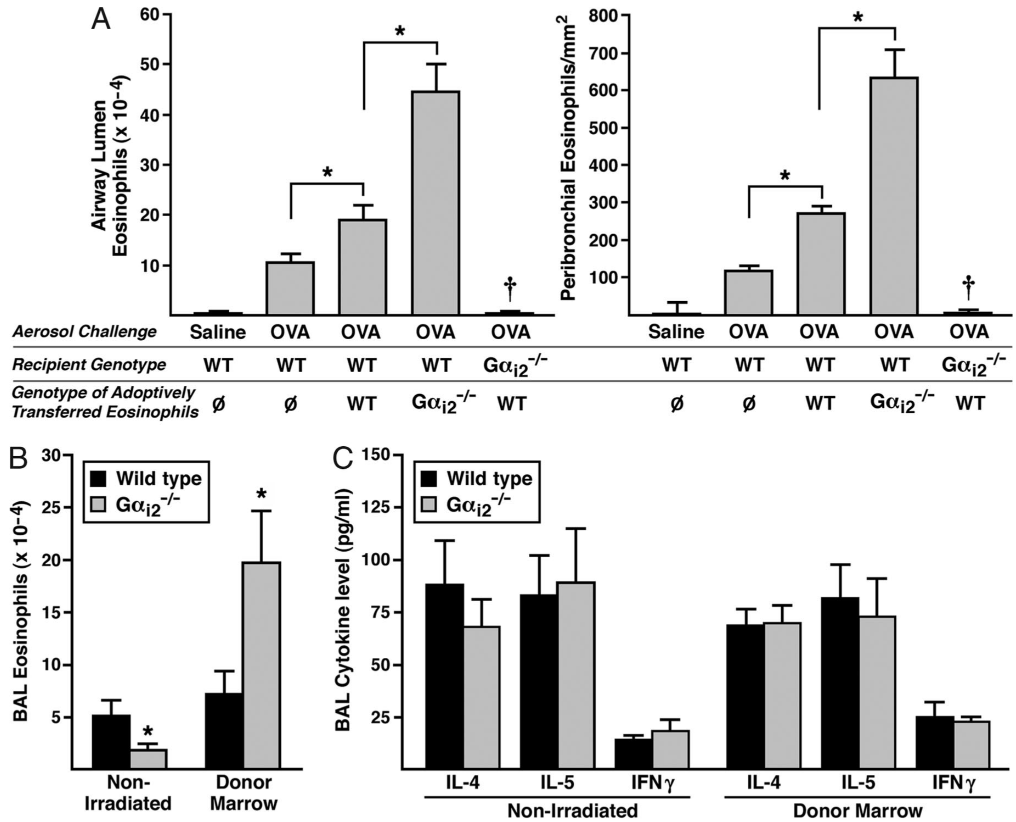

Fig. 2. $G\alpha_{i2}$ signaling in a non-lymphohematopoietic compartment(s) in the lung is necessary for eosinophil accumulation after allergen provocation. (*A*) Adoptive transfer of eosinophils into OVA-treated wild-type recipients ($n = 8$–10 mice per group) demonstrated that $G\alpha_{i2}{}^{-/-}$ eosinophils have an increased ability to traffic to both the airway lumen and the peribronchial areas of the lung. In contrast, the reciprocal transfer of wild-type eosinophils into OVA-treated $G\alpha_{i2}{}^{-/-}$ recipients demonstrated that eosinophil accumulation depended on lung-associated $G\alpha_{i2}$ signaling. ∅, transfer of PBS vehicle alone. ⋆, $P < 0.05$; †, significantly different ($P < 0.05$) from all other OVA groups examined. (*B*) Bone marrow engraftment of wild type recipients (Donor Marrow) demonstrated that the lack of eosinophil recruitment to the lung observed in $G\alpha_{i2}{}^{-/-}$ mice (Non-Irradiated) was a consequence of a $G\alpha_{i2}$ signaling event(s) in a lung structural cell type(s); $n =$ 10–12 mice per group, ⋆, $P < 0.05$. (*C*) OVA-induced pulmonary Th2 cytokines and IFN-γ levels in $G\alpha_{i2}{}^{-/-}$ mice are unaffected relative to OVA-treated wild type (Non-Irradiated). Moreover, OVA-induced Th2 cytokine and IFN-γ levels in OVA-treated mice after engraftment of $G\alpha_{i2}$ marrow into wild-type recipients was also unaffected relative to OVA-treated wild-type controls (Donor Marrow). All values presented are means ± SEM (n = 8 mice per group).

engraftment of wild-type marrow into $G\alpha_{i2}{}^{-/-}$ recipient mice have been unsuccessful (i.e., the mice die of an apparent septicemia within 5–7 days of engraftment), suggesting that $G\alpha_{i2}$ signaling events in the non-lymphohematopoietic tissues of these knockout recipients may be necessary for the recruitment and incorporation of stem cells in the marrow compartments of these mice.

Our studies involving the transfer of eosinophils indicated that the lack of pulmonary eosinophils during asthma in $G\alpha_{i2}{}^{-/-}$ mice was not due to a lack of production of eosinophils. Furthermore, assessments of eosinophilopoiesis in the bone marrow of $G\alpha_{i2}{}^{-/-}$ animals showed no effect on the production of circulating eosinophils in allergen naïve and allergen sensitized/aerosol-challenged mice (data not shown). However, leukocyte counts and cell differentials did show that eosinophils "backed-up" in the peripheral blood of $G\alpha_{i2}{}^{-/-}$ mice, accumulating to higher levels in the peripheral blood of both allergen naïve and OVA-treated animals (Fig. 3*A*). Moreover, and in agreement with published observations (14), this increased accumulation extended equally to all white blood cells, each differentially accumulating to higher levels in $G\alpha_{i2}{}^{-/-}$ mice relative to wild-type control groups (Fig. 3*A*).

Endotoxin-Mediated Pulmonary Accumulation of Neutrophils also Occurs as a Function of a $G\alpha_{i2}$-Signaling Pathways in a Resident Cell of the Lung. To extend our studies on the role of $G\alpha_{i2}$ in leukocyte migration to the lungs, we next assessed the recruitment and tissue accumulation of neutrophils as a function of an innate response to endotoxin; a model system that has been exploited in pulmonary studies by intranasal administration of LPS (see, for example, ref. 15). As with eosinophils, neutrophils have been previously characterized as expressing both $G\alpha_{i2}$ and $G\alpha_{i3}$ subunits (16), but the accumulation of these leukocytes in the lung after LPS administration was different in $G\alpha_{i2}$ vs. $G\alpha_{i3}$ knockout mice. The loss of $G\alpha_{i2}$ nearly abolished the LPS-induced pulmonary neutrophilia (Fig. 3*B*), whereas the neutrophilia induced in $G\alpha_{i3}$-deficient mice was unaffected relative to wild-type animals (Fig. 3*B*). Despite the loss of LPS-induced neutrophil accumulation in the lungs of $G\alpha_{i2}{}^{-/-}$ mice, this phenomenon did not appear to be a consequence of reduced chemotaxis resulting from the $G\alpha_{i2}$ deficiency in the neutrophil itself. *In vitro* assessments of chemotaxis by using transwell assays (Fig. 3*C*) demonstrated that $G\alpha_{i2}$-deficient neutrophils were capable of migrating in response to a ligand for the $G\alpha_i$-coupled receptor CXCR2 (MIP-2; ref. 17). Interestingly, *in vitro* MIP-2-mediated chemotaxis of $G\alpha_{i2}$-deficient neutrophils appeared to be enhanced relative to wild-type controls, consistent with our observations of eosinophils and previous studies by others investigating leukocytes deficient in genes mediating G protein-coupled receptor signal transduction (18). The similarity of the data examining both neutrophil and eosinophil recruitment again supports the leukocyte-independent character of the $G\alpha_{i2}$ deficiency in knockout mice. Further, the accumulation of leukocytes in the blood suggests a deficiency at an early stage in

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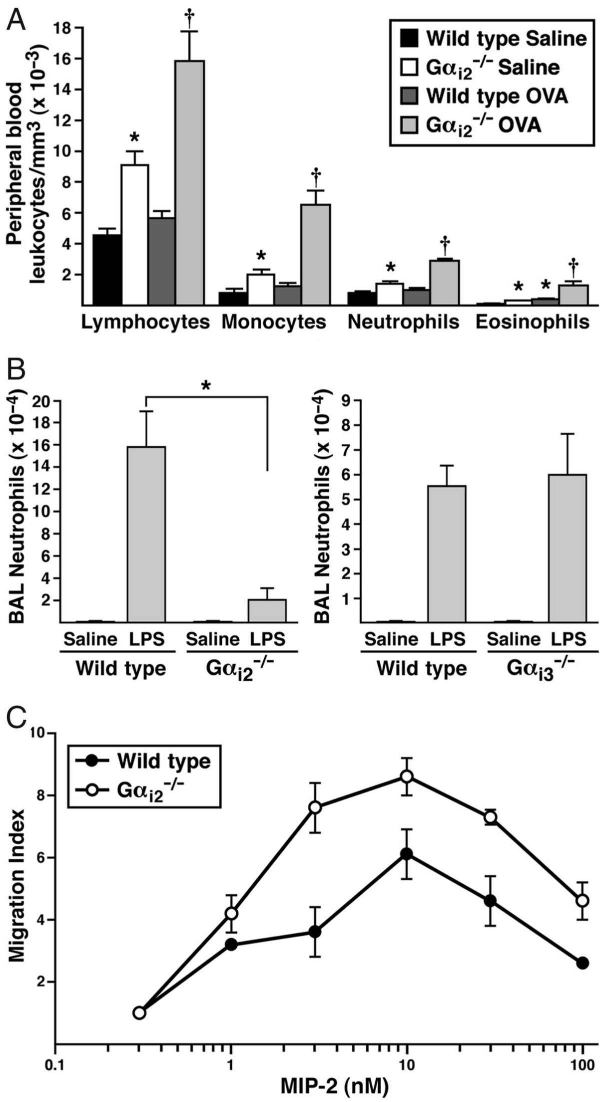

Fig. 3. The loss of $G\alpha_{i2}$ signaling in knockout mice leads to nonspecific increases in all circulating white blood cell types and severely limits LPS-induced airway neutrophil accumulation. (*A*) Increase in all white blood cell types is observed in both allergen-naïve and OVA-treated $G\alpha_{i2}{}^{-/-}$ mice. The data presented represent means ± SEM ($n = 5$ mice per group). *, significantly different ($P < 0.05$) from wild-type saline control mice. †, significantly different ($P < 0.05$) from OVA-treated wild-type mice. (*B*) LPS administered to $G\alpha_{i2}{}^{-/-}$ or $G\alpha_{i3}{}^{-/-}$ mice ($n = 7$–10 mice per group; wild-type animals served as negative controls) showed that the induced BAL neutrophil levels 16 h after administration were significantly decreased in $G\alpha_{i2}{}^{-/-}$ mice but unaffected in $G\alpha_{i3}{}^{-/-}$ animals. *, $P < 0.05$. (*C*) Transwell chemotaxis assays demonstrated that in the absence of $G\alpha_{i2}$ signaling events, *in vitro* neutrophil migration to MIP-2 was not lower but instead nominally higher relative to wild type.

cell exodus from the circulation. Once out of the vasculature, the leukocytes appear to have the ability to traffic along chemoattractant gradients and accumulate within specific tissue compartments.

Leukocyte Diapedesis and, in Turn, Tissue Accumulation Occurs as a Function of a $G\alpha_{i2}$-Signaling Event(s) in Endothelial Cells. Because our results suggested a leukocyte-independent impairment early in leukocyte trafficking from the blood, we next used intravital microscopy to examine endothelial cell–leukocyte interactions to determine whether a $G\alpha_{i2}$ deficiency in endothelial cells alters interactions with leukocytes in such a way as to limit extravasation. Visualization of abdominal mesenteric postcapillary venules from wild-type and $G\alpha_{i2}{}^{-/-}$ mice (Fig. 4*A* and SI Movie 1) after systemic i.p. administration of LPS [10 μg in 100 μl of phosphate-buffered saline (PBS)] demonstrated enhanced accumulation of stationary leukocytes on the endothelial surfaces in $G\alpha_{i2}{}^{-/-}$ animals relative to wild-type mice. Specifically, quantitative assessments of the leukocytes adhering to the venules demonstrated that whereas LPS exposure of wild-type mesentery induced only a nominal increase in static adherence to the underlying endothelium, the loss of $G\alpha_{i2}$ led to a >7-fold increase in the number of immobilized leukocytes (Fig. 4*A* and SI Fig. 10). This greater number of stationary leukocytes in knockout mice could be a consequence of enhanced cell adhesion mediated by the absence of a $G\alpha_{i2}$-signaling event(s) in the endothelium. Alternatively, the loss of $G\alpha_{i2}$ in the endothelium of knockout mice may slow down or block diapedesis, increasing the steady-state number of static endothelial-bound leukocytes which "back-up" on the endothelial surface. Our observation that all leukocyte subtypes accumulate in the blood of $G\alpha_{i2}{}^{-/-}$ mice, together with data demonstrating that different leukocyte subtypes use diverse repertoires of receptor–ligand interactions to mediate adhesion, suggest that a $G\alpha_{i2}$-dependent effect on cell adhesion is unlikely. This conclusion was confirmed by using *ex vivo* laminar flow assays to assess lymphocyte adherence and migration through an endothelial cell monolayer (Fig. 4 *B* and *C*). In these studies, $G\alpha_i$-signaling events were abolished in the endothelial cell monolayer by pretreatment with PTX. This pretreatment of the endothelial cells had no effect on cytotoxicity/viability or surface expression levels of the vascular cell adhesion molecule 1 (VCAM-1) (SI Figs. 11 and 12, respectively). The laminar flow study showed that α_4-integrin/VCAM-1-dependent firm adhesion of lymphocytes was unaffected in the absence of endothelial cell $G\alpha_i$ signaling (Fig. 4*B*), suggesting that effects on adhesion mediated by endothelial $G\alpha_{i2}$ signaling were not responsible for the blockade of leukocyte recruitment in knockout mice. However, the subsequent migration of firmly adherent lymphocytes through the endothelial cell monolayer (i.e., transmigration) in the laminar flow assay showed that this process was significantly inhibited after PTX treatment (i.e., a $G\alpha_i$-dependent event) of the monolayer (Fig. 4*C*). This result suggests that a signaling event(s) in the venule endothelium of $G\alpha_{i2}{}^{-/-}$ mice specifically is required for efficient transmigration/diapedesis.

Activated leukocyte trafficking and, in particular, the specificity involved in the extravasation cascade, requires a complex exchange of signals ("handshakes") between the mobile activated leukocytes and underlying vascular endothelial cells. The general responses of leukocytes and endothelial cells to chemokines have been studied (19) as has the role of adhesion molecules and their receptors in the trafficking response (20). The involvement of $G\alpha_i$-coupled receptors in leukocyte activation, rolling, and subsequent arrest at the endothelial surface before diapedesis has been shown to involve a PTX-sensitive step at the level of the leukocyte (e.g., $G\alpha_i$-coupled chemokine-mediated recruitment). However, our data also suggest that the complex interaction between endothelial cells and leukocytes responding to inflammatory signals includes a signal transduction event involving the $G\alpha_{i2}$ isoform in endothelial cells, which leads to the extravasation of circulating leukocytes. The activation of this pathway may occur in several different ways. For example, the interaction of endothelial cells with leukocytes activates a variety of ectoenzymes. Thus, transmigration may require a $G\alpha_{i2}$-mediated response in the endothelial cells to signals or ligands generated by these ectoenzymes (reviewed in ref. 21). Alternatively, local changes in nitric oxide have been shown to modify the levels of RGS (Regulators of G protein Signaling) proteins that are required for regulating $G\alpha_{i2}$-signaling events in endothelial cells (22). Moreover, "outside-in"

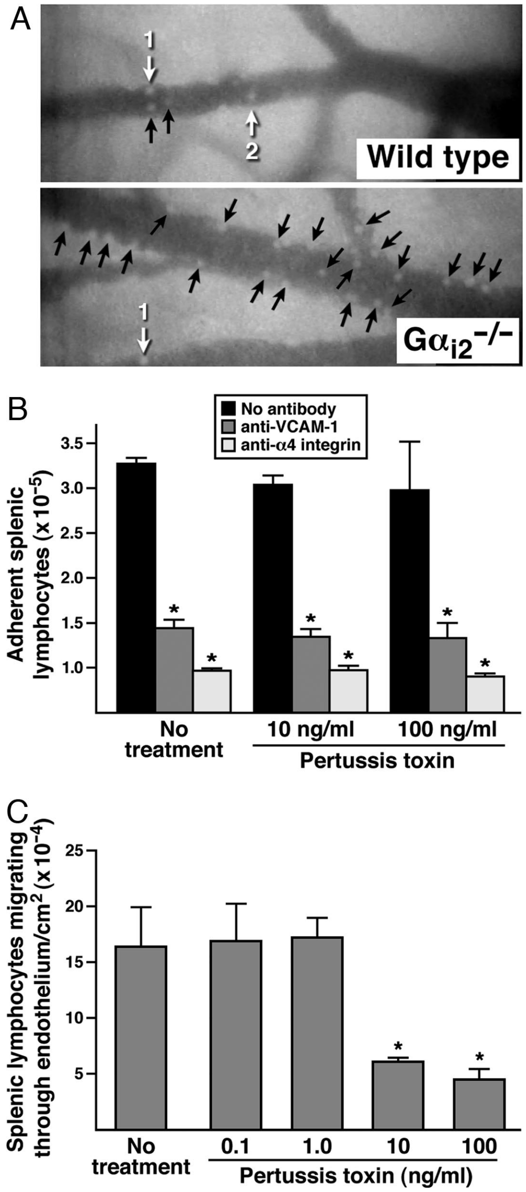

Fig. 4. The loss of $G\alpha_{i2}$ signaling in the vascular endothelium leads to the accumulation of immobilized cells in postcapillary venules through a blockade of diapedesis. (*A*) Intravital photomicroscopy of LPS-exposed mesentery postcapillary venules showed that the loss of $G\alpha_{i2}$ resulted in a significant increase of stationary leukocytes adherent to the vascular endothelium. Numbered white arrows indicate individual rolling leukocytes and black arrows identify stationary leukocytes. The $G\alpha_i$-dependence of lymphocyte binding and migration through an endothelial cell monolayer was assessed by using an *ex vivo* parallel plate flow chamber under conditions of laminar flow comparable to pressures observed in postcapillary venules [i.e., 2 dynes (1 dyne = 10 μN)/cm^2]. (*B*) VCAM-1-dependent firm adhesion was blocked by antibodies against either endothelial VCAM-1 or lymphocyte associated α_4-integrin but was unaffected by PTX pretreatment of the endothelial cell monolayer. *, significantly different ($P < 0.05$) from no-antibody control group. (*C*) Lymphocyte migration through an endothelial cell monolayer is blocked in a concentration-dependent fashion by pretreatment of the endothelial cells with PTX. "No treatment," endothelial cells treated with PBS. *, significantly different ($P < 0.05$) from PBS-treated control group.

$G\alpha_{i2}$-mediated signaling events in endothelial cells leading to the release and movement of previously stationary leukocytes may occur through the mobilization and activation of intercellular adhesion molecule/VCAM-tetraspanin adhesion complexes (23) or the regulation of endothelial integrity through Pky2-mediated affects on vascular endothelial-cadherin adhesion (24). Regardless of the specific mechanism(s) mediating tissue accumulation of leukocytes, our data have surprisingly shown that a $G\alpha_{i2}^{-/-}$-dependent endothelial cell signaling event(s) is required for efficient diapedesis. Equally surprising was the fact that the overlapping functions of other $G\alpha_i$ family members that are generally capable of compensating for the loss of $G\alpha_{i2}$ in leukocytes are not able to do so in the endothelial cells.

These observations have clinical implications and may offer new possibilities for the treatment of inflammatory diseases. In particular, the observation that an endothelial cell-specific signal transduction event(s) is required for the extravasation of multiple proinflammatory leukocyte subtypes suggests that this could be a rate-limiting step regulating their tissue accumulation in a myriad of diseases. Moreover, the events leading to diapedesis may be the result of a number of endothelial cell receptors that are coupled only to $G\alpha_{i2}$-containing G proteins. Therefore, these receptors may potentially represent targets for drug intervention that are potentially applicable to a wide variety of both chronic and acute inflammatory diseases.

Materials and Methods

Mice. $G\alpha_{i2}$ and $G\alpha_{i3}$-null mice were generated by homologous recombination in embryonic stem cells (background strain: 129) as described (25). Subsequent generations of G protein-deficient animals were the result of backcrosses ($>F_5$) onto the inbred strain C57BL/6J, a strain that does not display the baseline inflammatory responses observed in the original 129 knockout animals (25). Eosinophils were isolated from $G\alpha_{i2}^{-/-}$ or $G\alpha_{i3}^{-/-}$ mice constitutively expressing mouse IL-5 that resulted from crosses of the respective G protein-deficient animals with the IL-5 transgenic line NJ.1638 [C57BL/6J (>20 backcross generations)] (13).

Induction of Allergic Airway Inflammation. The OVA model of allergic pulmonary inflammation has been described (26). For details see *SI Materials and Methods*.

LPS-Induced Airway Inflammation. Mice were lightly anesthetized with isoflurane before intranasal administration of 10 μg (20 μl total volume) of LPS (Sigma, St. Louis, MO). The number of neutrophils recruited to the airways 16 h post-LPS administration were determined by BAL, modifying a described protocol (27). BAL fluid was recovered following instillation of saline supplemented with 2% FCS (1 ml). The BAL fluid was centrifuged at low speed (10 min at 400 $\times$ *g*; 4°C). The recovered cells were counted by using a hemocytometer, and cell differentials were performed on stained cytospin preparations (Diff-Quik; Dade Behring, Newark, DE) counting >300 cells.

Lung Histology and Immunohistochemistry. Lung tissue for histological analysis was obtained by instilling ≈1 ml of 10% neutral-buffered formalin (30 cm H_2O constant pressure) through a cannula inserted into the trachea. The excised lung was immersed in formalin for 24 h (at 4°C). Parasagittal sections (4 μm) were obtained from paraffin-embedded tissue, stained with hematoxylin and eosin, and analyzed by bright field microscopy. Eosinophil recruitment to lung tissue compartments was determined by immunohistochemistry using a rabbit polyclonal anti-mouse major basic protein (MBP) antisera (28). Eosinophils surrounding the airways were quantified by counting the number of MBP-positive cells per square millimeter of submucosal tissue surrounding the bronchioles.

Isolation of Mouse Eosinophils, Isolation of Splenocytes, and *In Vitro* Assessment of Leukocyte Chemotaxis. For details, see *SI Materials and Methods*.

Eosinophil Adoptive Transfer and *Ex Vivo* Labeling of Eosinophils and Monitoring the Recruitment/Accumulation of Labeled Eosinophils Following Adoptive Transfer to the Peritoneal Cavity. For details, see *SI Materials and Methods*.

Hematopoietic Engraftment by Bone Marrow Transfer. Exposing female wild-type mice to 1,100-cGy whole body lethal irradiation generated complete bone marrow chimeras. Within 3 h of irradiation, 1×10^7 bone marrow cells from wild-type or $G\alpha_{i2}{}^{-/-}$ male donors were transferred by tail vein injection. Engrafted mice were used in experiments after a >45-day recovery period. Recovered mice were sensitized and aerosol challenged with OVA (saline for controls) by using the protocol noted above, and BAL eosinophils were enumerated 24 h after the last challenge. Eosinophils comprise <1% of leukocytes in the airways of saline-challenged mice of any group. Donor cell engraftment of >90% was achieved in all recipients as determined by a PCR assay designed to quantify X vs. Y chromosome-specific sequences (29).

Cytokine Assays. Cytokine levels in BAL fluid were determined by ELISA. Mouse IL-4, IL-5, IFN-γ, and IL-12 ELISA kits (R&D Systems, Minneapolis, MN) were used according to the manufacturer's protocol. The limits of detection for each assay were as follows: IFN-$\gamma \approx 30$ pg/ml, IL-4 ≈ 10 pg/ml, IL-5 ≈ 10 pg/ml, and IL-12 ≈ 10 pg/ml.

***In Vivo* Assessment of Leukocyte Intracapillary Rolling and/or Adhesion in Postcapillary Venules by Intravital Microscopy and *Ex Vivo* Assessments of Lymphocyte Adhesion and Migration.** For details, see *SI Materials and Methods*.

Determination of VCAM-1 Endothelial Cell Expression Using Flow Cytometry. mHEVa endothelial cell monolayers (before or after exposure to PTX) were disassociated into single-cell suspensions by using 0.3% EDTA, and the recovered cells were washed in RPMI-1640 containing 20% FCS. Cells were stained in PBS/0.5% BSA/0.15% NaN_3 with rat anti-mouse VCAM-1 antibody (normal rat IgG was used as a negative control for primary antibody staining) and visualized with biotin-conjugated goat anti-rat antibodies and strepavidin-phycoerythrin. Analysis was performed on a FACScan flow cytometer (BD Biosciences, Palo Alto, CA) with CellQuest Pro software (BD Biosciences).

Statistical Analysis. All data presented are the means ± standard errors (SEM). Statistical analysis was performed on parametric data by using *t* tests with differences between means considered significant when $P < 0.05$.

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