Identification of an alternative $\text{G}_q$-dependent chemokine receptor signal transduction pathway in dendritic cells and granulocytes

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CD38 controls the chemotaxis of leukocytes to some, but not all, chemokines, suggesting that chemokine receptor signaling in leukocytes is more diverse than previously appreciated. To determine the basis for this signaling heterogeneity, we examined the chemokine receptors that signal in a CD38-dependent manner and identified a novel "alternative" chemokine receptor signaling pathway. Similar to the "classical" signaling pathway, the alternative chemokine receptor pathway is activated by $\text{G}_q$-containing $\text{G}_i$ proteins. However, unlike the classical pathway, the alternative pathway is also dependent on the $\text{G}_q$ class of $\text{G}$ proteins. We show that $\text{G}_q$-deficient neutrophils and dendritic cells (DCs) make defective calcium and chemotactic responses upon stimulation with N-formyl methionyl leucyl phenylalanine and CC chemokine ligand (CCL) 3 (neutrophils), or upon stimulation with CCL2, CCL19, CCL21, and CXC chemokine ligand (CXCL) 12 (DCs). In contrast, $\text{G}_q$-deficient T cell responses to CXCL12 and CCL19 remain intact. Thus, the alternative chemokine receptor pathway controls the migration of only a subset of cells. Regardless, the novel alternative chemokine receptor signaling pathway appears to be critically important for the initiation of inflammatory responses, as $\text{G}_q$ is required for the migration of DCs from the skin to draining lymph nodes after fluorescein isothiocyanate sensitization and the emigration of monocytes from the bone marrow into inflamed skin after contact sensitization.

Over the last 15 yr, many of the key intracellular proteins and second messengers that control cell migration have been identified, and a consensus chemokine receptor signal transduction model has been proposed (1). One of the critical components of this chemokine receptor signaling model is the trimeric $\text{G}$ protein complex that directly associates with chemokine receptors and transduces signals from these receptors to other key intracellular signaling molecules (2–5).

The $\text{G}$ proteins associated with chemokine receptors contains three subunits: $\text{G}_q$, $\beta$, and $\gamma$. The activation of $\text{G}$ proteins is induced by the binding of GTP to $\text{G}_q$ and the release of free $\beta\gamma$. This initiates the chemokine receptor signaling cascade, with the free $\beta\gamma$ subunits activating downstream effectors (6, 7) like phosphoinositide 3-kinase 3–kinase that control cytoskeletal changes necessary for chemotaxis (8–10). In addition, the $\beta\gamma$ subunits released from chemokine receptor–associated $\text{G}$ proteins are capable of activating phospholipase (PLC) $\beta_2$ and PLC$\beta_3$ (11, 12), the enzymes responsible for inositol trisphosphate (IP$_3$) production and intracellular calcium release from IP$_3$-gated stores (13). Inhibitors of $\text{G}_i$, such as pertussis toxin (PTx), block all of these downstream signaling events (14–16) and inhibit the in vitro and in vivo migration of effectively all leukocyte populations.

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(for review see reference 17). Thus, G proteins are of central importance in leukocyte trafficking.

Recently, we identified the ectoenzyme CD38 as another important signaling protein in the chemokine receptor signaling pathway (18). CD38, a nicotinamide adenine dinucleotide (NAD) glycohydrolase and ADP-ribosyl cyclase (19), is expressed by many leukocyte populations, including neutrophils, monocytes, DCs, and lymphocytes (20, 21). The extracellular enzymatic domain of CD38 catalyzes the formation of cyclic adenosine diphosphoribose (cADPR), ADPR, and nicotinic acid adenine dinucleotide from its substrates NAD\(^+\) and NADP\(^+\) (22). All three products of the CD38 enzyme reaction function as signaling molecules either by mobilizing calcium from intracellular stores (23) or by activating calcium entry from the extracellular space (24–27). We showed that at least one of the metabolites made by CD38, cADPR, controls calcium influx in chemokine-stimulated cells, and that this CD38- and cADPR-dependent calcium response is required for neutrophil and DC migration both in vitro and in vivo (28, 29). Furthermore, loss of CD38-dependent chemokine receptor signal has important immunological consequences, as mice deficient in CD38 (Cd38\(^{−/−}\)) are more susceptible to bacterial infections (28) and make attenuated innate and adaptive immune responses to inflammatory agents and immunogens (29, 30).

Based on these data, we concluded that CD38 is a critical regulator of cell migration. However, unlike G\(\alpha_q\)-containing G proteins, which are required for the chemotaxis of virtually all hematopoietic cells to all chemoattractants (17), CD38 is not universally required for cell migration. Instead, we found that CD38 is required for leukocyte chemotaxis to some, but not all, chemokines (28, 31). These data suggested that the signals required to induce chemotaxis have to be more diverse than previously appreciated, and we hypothesized that assessing CD38-dependent pathways would allow us to identify new proteins involved in cell migration. In this study, we directly tested this hypothesis and discovered a novel “alternative” chemokine receptor signaling pathway that is required for cell chemotaxis. Similar to the “classical” chemokine receptor signaling pathway, the alternative chemokine receptor signaling pathway is dependent on G\(\alpha_q\). However, unlike the classical chemokine receptor signaling pathway, the alternative pathway is also dependent on G\(\alpha_q\)-containing G proteins. Despite the ability of G\(\alpha_q\) proteins to directly activate several isoforms of PLC\(\beta\) (32, 33), G\(\alpha_q\) is not required for chemokine-induced IP\(_{3}\)-mediated intracellular calcium release. Instead, G\(\alpha_q\), like CD38, regulates extracellular calcium entry in chemokine-stimulated cells. Importantly, we found that G\(\alpha_q\)-deficient (Gnaq\(^{−/−}\)) DCs and monocytes are unable to migrate to inflammatory sites and LNs in vivo, demonstrating that this alternative G\(\alpha_q\)-coupled chemokine receptor signaling pathway is critically important for the initiation of immune responses. The implications of these unexpected findings for chemokine receptor biology and inflammation are discussed.

RESULTS

Chemokine receptors can be divided into CD38-dependent and -independent subclasses

We previously showed that the in vitro chemotaxis of mouse formyl peptide receptor (mFPR) 1–expressing bone marrow neutrophils to the chemoattractant N-formyl methionyl leucyl phenylalanine (fMLF) is dependent on CD38 and at least one of its enzymatically generated products, cADPR (28, 31). Indeed, neither WT neutrophils pretreated with the membrane-permeant cADPR antagonist 8Br-cADPR nor Cd38\(^{−/−}\) neutrophils migrate in transwell chambers in response to an optimal concentration of fMLF (Fig. 1 A). In contrast, chemotaxis of Cd38\(^{−/−}\) neutrophils and 8Br-cADPR–treated WT neutrophils to the CXC chemokine receptor (CXCR) 1/CXCR2 ligand IL-8 (Fig. 1 B) is equivalent to chemotaxis of normal WT neutrophils, indicating that there must be at least two subclasses of chemokine receptors that can be distinguished from one another based on their requirement for CD38.

In subsequent analyses of mouse and human leukocytes, we found additional examples of chemokine receptors that signal in either a CD38/cADPR-dependent or -independent manner (28, 29, 31). However, when we compared the chemotactic response of peripheral (isolated from spleen and LNs) mouse DCs and T cells with the same CC chemokine receptor (CCR) 7 ligand, CCL19, we found that the peripheral Cd38\(^{−/−}\) DCs were unable to migrate in response to the CCL19 gradient (Fig. 1 C), whereas CD4 T cells purified from the same tissues of the same Cd38\(^{−/−}\) mice migrated normally in response to CCL19 (Fig. 1 D). Likewise, peripheral WT DCs pretreated with the cADPR antagonist 8Br-cADPR made a defective chemotactic response to CCL19 (Fig. 1 C), whereas the chemotactic response of WT T cells pretreated with 8Br-cADPR was equivalent to that observed for the untreated WT T cells (Fig. 1 D). Collectively, these data showed that chemokine receptors can be divided into different subclasses and that the subclass of the chemokine receptor is variable and dependent on the cell type expressing the chemokine receptor.

CD38-dependent chemokine receptors couple to G\(\alpha_q\)

Our data suggested that there was considerably more diversity or heterogeneity in the molecular signals that regulate chemotaxis than previously appreciated. To better understand the diversity between chemokine receptors, we examined the response of WT and Cd38\(^{−/−}\) neutrophils to platelet-activating factor (PAF), a ligand of the PAFR. We chose to analyze signaling through this receptor, as it is one of the few known chemoattractant receptors that can induce calcium release in a PTx-independent fashion, indicating that it must functionally couple to other G proteins in addition to those containing G\(\alpha_q\) (34–36). Therefore, we loaded bone marrow WT and Cd38\(^{−/−}\) neutrophils with calcium-sensing fluorescent dyes, stimulated the cells with PAF, and measured accumulation of intracellular free calcium by FACS. As previously reported for human neutrophils (34), PAF-stimulated

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human neutrophils (34), the immediate calcium release from IP3-gated intracellular stores was reduced, although not absent, in the PAF-stimulated Gnaq−/− neutrophils relative to WT neutrophils (Fig. 2 A). Furthermore, in a manner identical to what we observed with Cd38−/− neutrophils, the PAF-induced calcium response was not sustained in the Gnaq−/− neutrophils (Fig. 2 A), suggesting that Gαq regulates both intracellular calcium release and calcium influx in these cells.

These results suggested that CD38 and Gαq might both be involved in activating calcium influx in chemokine-stimulated cells. If this conclusion is correct, then we postulated that Gαq, like CD38, might also regulate calcium influx in fMLF-stimulated neutrophils. To test this hypothesis, we measured the calcium response of fMLF-activated bone marrow neutrophils isolated from WT, Cd38−/−, and Gnaq−/− mice. As shown earlier (28, 31), we observed a bimodal calcium response in the fMLF-activated WT neutrophils and a significantly reduced calcium response in the fMLF-stimulated Cd38−/− neutrophils (Fig. 2 B). Interestingly, the calcium response of the fMLF-activated Gnaq−/− neutrophils was very similar to that of the Cd38−/− neutrophils and significantly reduced relative to the WT neutrophils (Fig. 2 B). We observed the most pronounced reduction in the calcium response of the fMLF-activated Gnaq−/− neutrophils during the second calcium influx phase of the response (28), suggesting a

WT bone marrow neutrophils made a bimodal calcium response with a rapid rise in intracellular free calcium levels that was followed by a second phase of sustained calcium mobilization (Fig. 2 A). The first phase of calcium release was largely caused by calcium release from intracellular stores, as it was not blocked in the presence of EGTA, whereas the second phase was caused by calcium entry, as it was inhibited when EGTA was added to the external medium (unpublished data). Interestingly, the first calcium release from intracellular stores was intact in the PAF-activated Cd38−/− neutrophils; however, minimal calcium entry was observed during the second sustained phase of the response (Fig. 2 A). Similar results were observed with 8Br-cADPR–treated WT neutrophils and with PAF-stimulated bone marrow–derived Cd38−/− DCs (unpublished data). Thus, calcium signaling through the PAFR appears to be regulated by CD38 and cADPR.

It has been previously demonstrated that PTx-treated PAF-activated cells produce IP3 (34–36), indicating that the PAFR must couple to one or more members of the Gq family of G proteins that are capable of activating PLCβ and inducing IP3 formation (32, 33). To assess whether the PAFR couples to Gαq, we purified bone marrow neutrophils from chimeric mice that lacked Gαq (Gnaq−/−) expression in the bone marrow–derived hematopoietic cells. We then stimulated the cells with PAF and measured intracellular calcium accumulation. Similar to the previous experiments that used PTx-treated

Figure 1. Differential control of leukocyte chemotaxis by the CD38/cADPR signaling pathway. (A and B) Bone marrow neutrophils from C57BL/6J (WT and WT + 8Br-cADPR) and Cd38−/− mice were preincubated for 20 min in media (white and black bars) or 100 μM 8Br-cADPR (gray bars) and placed in transwell chambers containing media (nil), or 1 μM fMLF (A) or 100 nM IL-8 (B) in the bottom chamber. The cells that migrated to the bottom chamber in response to the chemokine gradient were collected after 1 h and enumerated by FACS. (C and D) Splenic and LN CD11c+ DCs and splenic CD4+ T cells were purified from WT and Cd38−/− mice. The DCs (C) and T cells (D) were preincubated for 20 min in media or 8Br-cADPR (as described for A and B) and placed in transwell chambers containing media or CCL19 (50 ng/ml for DCs and 300 ng/ml for T cells). The number of cells that migrated to the bottom chamber after 2 h was determined by FACS. The results are expressed as the mean ± SD of triplicate cultures. The data shown are representative of four or more independent experiments. *, P < 0.0007 between WT cells and the indicated groups. ns, not significant.
Figure 2. Signaling through CD38-dependent chemokine receptors also requires Gnaq. (A–C) Bone marrow neutrophils from WT (blue), Cd38−/− (green), and Gnaq−/− (red) mice were loaded with the calcium-detecting dyes Fluo-3 and Fura-red and stimulated with 100 nM PAF (A), 1 μM fMLF (B), or 100 nM IL-8 (C). Relative intracellular calcium levels were measured by FACS and are reported as the ratio of Fluo-3/Fura-red. Arrows indicate when the stimulus was added to the cells. (D and E) Bone marrow neutrophils from WT and Gnaq−/− mice were placed in transwells containing media (nil), fMLF (D, 1 μM; E, 0.1–5 μM), 100 nM IL-8, or 50 ng/ml CCL3. The cells that migrated to the bottom chamber in response to the chemokine gradient were collected after 1 h and enumerated by FACS. The results are expressed as the mean ± SD of the CI (see Materials and methods for description) of triplicate cultures. The data shown are representative of four or more independent experiments. *, P ≤ 0.0001; or **, P < 0.03 between WT and Gnaq−/− neutrophils.

prominent role for Gαq in regulating calcium entry rather than intracellular calcium release in this response. To bolster this conclusion, we next examined the calcium response of IL-8–activated Gnaq−/− neutrophils, as the calcium response to IL-8 in mouse bone marrow neutrophils is almost entirely caused by IP3-induced intracellular calcium release (28). In agreement with our hypothesis, the calcium response of IL-8–stimulated neutrophils was largely unaffected by the loss of Gαq (Fig. 2 C), again very similar to what we observed with the Cd38−/− neutrophils (Fig. 2 C).

Collectively, these data suggested a strong positive correlation between CD38 and Gαq, at least with respect to calcium influx in response to chemokine receptor ligation. Given the requirement for both CD38 and calcium influx in neutrophil chemotactic responses to chemoattractants such as fMLF (28), we postulated that Gαq was also likely to be required for chemotaxis to chemokines that signal in a CD38–dependent fashion. To test this possibility, we examined the in vitro chemotaxis of Gnaq−/− bone marrow neutrophils to chemokines that signal in a CD38– and calcium influx–independent (IL-8) or a CD38– and calcium influx–dependent (fMLF and CCL3) fashion (28). As shown in Fig. 2 D, in vitro chemotaxis of the Gnaq−/− neutrophils to IL-8 was completely normal. However the Gnaq−/− cells were unable to migrate in response to either fMLF or CCL3 (Fig. 2 D). This impaired chemotactic response was not caused by an altered responsiveness to the chemoattractants, as the Gnaq−/− neutrophils made a defective chemotactic response at all doses of fMLF (see Fig. 3 E) or CCL3 (not depicted) tested. Instead, the data demonstrate the existence of a novel Gαq− and CD38–dependent chemokine receptor signaling pathway that is engaged by some, but not all, chemokine receptors.

Signaling through a subset of chemokine receptors requires both Gαi and Gαq

The results showing that chemotaxis of neutrophils to mFPR1 and CCR1 ligands is Gαq dependent were surprising, as the Gi inhibitor PTx is known to block chemotaxis of essentially all leukocytes (17). Because we could not find any reports testing the effect of PTx on mouse bone marrow–derived neutrophils, we thought it important to test this assumption experimentally. Therefore, we treated WT neutrophils with PTx and then measured the calcium response to IL-8 and fMLF. Similar to published data (37), the calcium responses were largely, although not entirely, ablated in the PTx-treated bone marrow neutrophils stimulated with either IL-8 (Fig. 3 A) or fMLF (Fig. 3 B). To confirm the PTx results, we also measured intracellular calcium levels and chemotaxis in WT, Gαi2–deficient (Gnia2−/−), and Gnaq−/− neutrophils that were activated with either IL-8 or fMLF. Similar to the PTx-treated cells, Gnia2−/− neutrophils made a very reduced calcium response after exposure to either IL-8 or fMLF (Fig. 3, C and D). However, the calcium response of the Gnaq−/− neutrophils to IL-8 was normal (Fig. 3 C), and the calcium response of the Gnaq−/− cells to fMLF was only partially impaired (Fig. 3 D). Not surprisingly, Gnia2−/− neutrophils were unresponsive to both IL-8 and fMLF in the in vitro chemotaxis assay (Fig. 3 E). In contrast, the chemotactic response of
we examined the calcium response of chemokine-stimulated $G_{naq}/H_11002$ mouse bone marrow neutrophils in more detail. Initially, we stimulated $G_{naq}/H_11002$ neutrophils with fMLF in the presence or absence of EGTA (to chelate the extracellular calcium) so that we could assess the relative contribution of $G_{q}$ to intracellular calcium release and extracellular calcium influx. Identical to our earlier results, the calcium response of fMLF-stimulated $G_{naq}/H_11002$ neutrophils initiated apparently normally but was not sustained and rapidly dropped to background levels (Fig. 4A). Indeed, as shown in Fig. 4B, the calcium response of the EGTA-treated fMLF-activated $G_{naq}/H_11002$ neutrophils was very similar to that seen in equivalently treated WT neutrophils, indicating that $G_{q}$ is not obligatorily required for intracellular calcium release, at least in this cell type. Because intracellular calcium release in chemokine receptor–stimulated leukocytes is IP$_3$ dependent, we asked whether we could block the remaining calcium response in $G_{naq}/H_11002$ neutrophils by treating the fMLF-activated $G_{naq}/H_11002$ cells with the IP$_3$ inhibitor 2-aminoethoxydiphenylborate (2-APB). As shown in Fig. 4C, the calcium response of $G_{naq}/H_11002$ neutrophils to fMLF was ablated, whereas the response to IL-8 was completely intact (Fig. 3E). Collectively, the data indicate that some chemokine receptors expressed by mouse bone marrow neutrophils, such as CXCR1/2, couple to G proteins containing $G_{i2}$ but not $G_{q}$. However other receptors, such as mFPR1 and CCR1, couple to two different classes of G proteins, $G_{i2}$ and $G_{q}$.

$G_{q}$ regulates calcium influx in chemokine-stimulated neutrophils

The “signature” activity of the Gq class of G proteins is to activate one or more PLCβ isoforms, which catalyze production of IP$_3$ and promote intracellular calcium release (32, 33). This activity is performed by the α subunit of the trimeric Gq-containing proteins (32, 33). Therefore, it was surprising to find that the largest alteration in the calcium response of the fMLF-stimulated $G_{naq}/H_11002$ neutrophils appeared during the later calcium entry phase of the response rather than during the initiation of the calcium response. To better understand how $G_{q}$ regulates chemokine receptor signal transduction, we examined the calcium response of chemokine-stimulated $G_{naq}/H_11002$ mouse bone marrow neutrophils in more detail. Initially, we stimulated $G_{naq}/H_11002$ neutrophils with fMLF in the presence or absence of EGTA (to chelate the extracellular calcium) so that we could assess the relative contribution of $G_{q}$ to intracellular calcium release and extracellular calcium influx. Identical to our earlier results, the calcium response of fMLF-stimulated $G_{naq}/H_11002$ neutrophils was very similar to that seen in equivalently treated WT neutrophils, indicating that $G_{q}$ is not obligatorily required for intracellular calcium release, at least in this cell type. Because intracellular calcium release in chemokine receptor–stimulated leukocytes is IP$_3$ dependent, we asked whether we could block the remaining calcium response in $G_{naq}/H_11002$ neutrophils by treating the fMLF-activated $G_{naq}/H_11002$ cells with the IP$_3$ inhibitor 2-aminoethoxydiphenylborate (2-APB). As shown in Fig. 4C, the calcium response of $G_{naq}/H_11002$ neutrophils to fMLF was ablated, whereas the response to IL-8 was completely intact (Fig. 3E). Collectively, the data indicate that some chemokine receptors expressed by mouse bone marrow neutrophils, such as CXCR1/2, couple to G proteins containing $G_{i2}$ but not $G_{q}$. However other receptors, such as mFPR1 and CCR1, couple to two different classes of G proteins, $G_{i2}$ and $G_{q}$.

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The data shown are representative of at least three independent experiments.

In B, the extracellular calcium was chelated with 2 mM EGTA immediately before stimulation. Arrows indicate when the stimulus was added to the cells. The cells were loaded with Fluo-3 and Fura-red and stimulated with 1 μM fMLF. Relative intracellular calcium levels were measured by FACS and are reported as the ratio of fluo-3/Fura-red. In B, the extracellular calcium was chelated with 2 mM EGTA immediately before stimulation. Arrows indicate when the stimulus was added to the cells. The data shown are representative of at least three independent experiments.

Figure 4.  Gnaq and cADPR coregulate calcium influx in fMLF-stimulated neutrophils. (A–D) Bone marrow neutrophils from WT (blue) or Gnaq−/− (red, green, and black) mice were preincubated in media (Gnaq−/−, red; WT, blue), 100 μM 8Br-cADPR (green), or 100 μM 2-APB (black) for 20 min. The cells were loaded with Fluo-3 and Fura-red and stimulated with 1 μM fMLF. Relative intracellular calcium levels were measured by FACS and are reported as the ratio of fluo-3/Fura-red. The calcium response was ablated in the 2-APB-treated Gnaq−/− neutrophils, similar to that observed with fMLF-activated Gnaq−/− neutrophils (Fig. 3).

The calcium responses of the Gnaq−/− neutrophils were very similar to our previous findings using Cd38−/− neutrophils (28, 31), suggesting to us that Gαq and CD38 likely coregulate the same calcium entry pathway in the fMLF-activated neutrophils. To test this possibility, we stimulated Gnaq−/− neutrophils with fMLF in the presence or absence of the cADPR antagonist 8Br-cADPR and measured the calcium response. As predicted, the calcium signaling profiles were identical between the untreated and 8Br-cADPR-treated Gnaq−/− cells (Fig. 4 D). Collectively, the data indicate that Gαq controls the IP3-gated calcium release, and that Gαq and CD38 coordinately sustain the calcium response by activating calcium entry in fMLF-activated mouse bone marrow neutrophils. The data further suggest that the Gαq-dependent calcium release is necessary for the initiation of the sustained calcium response in the fMLF-activated bone marrow neutrophils. However, it is also possible that Gαi2 additionally regulates the CD38- and Gαq-dependent calcium influx response by other mechanisms unrelated to intracellular calcium release.

Gαq differentially regulates chemokine receptor signaling in DCs and T lymphocytes

Because Gαq and CD38 are both required for productive mFPR1 and CCR1 signaling in mouse neutrophils, we postulated that Gαq would also be required for chemotaxis of DCs to CXCR4 and CCR7 ligands, as signaling through these receptors is CD38 and cADPR dependent (Fig. 1). Furthermore, we predicted that signaling through CXCR4 and CCR7 in T cells would not be Gαq dependent, as CD38 is not required for the chemotaxis of these cells to the CXCR4 and CCR7 ligands CXCL12 and CCL19 (Fig. 1). Instead, we predicted that T cell chemotaxis to these ligands would require Gαi2. To test these predictions, we measured the in vitro chemotaxis of DCs and T cells from WT, Gnaq−/−, and Gnaq−/− mice to CCL19 and CXCL12. As expected based on previously published experiments using the Gi inhibitor PTx (38–40), the chemotaxis of T cells and DCs to both chemokines requires Gαq (Fig. 5, A and C). However, chemotaxis of Gnaq−/− T cells to the same two chemokines was completely normal (Fig. 5 B), indicating that CCR7 and CXCR4 signaling is Gαq, but not Gαi2, dependent in T cells. In striking contrast, the chemotaxis of Gnaq−/− DCs to CXCL12 and CCL19 was ablated (Fig. 5 D). The impaired chemotaxis of the Gnaq−/− DCs was not caused by defective chemokine receptor expression, at least for CXCR4, as this receptor was expressed at equivalent levels in the WT, Gnaq−/−, and Gnaq−/− DCs (Fig. 5 E). Thus, Gαq, like CD38, regulates CCR7 and CXCR4 signaling in DCs but not in T cells. Collectively, these data indicate that the same chemokine receptor can couple to the Gαq/CD38-dependent signaling pathway in one cell type but not necessarily in all cell types.

Inflammation-induced migration of DCs and monocytes requires Gαq

Previous results showed that Gαq regulates the in vitro chemotaxis of DCs but not B cells (not depicted) and T cells (Fig. 5). Based on these data, we predicted that Gαq would also be required for the in vivo migration of DCs to secondary lymphoid organs. To test this hypothesis, we first examined the composition and cellularity of the skin, LNs, and spleens of Gnaq−/− chimeras. As shown in Table I, the total cellularity of the various LNs (axillary and mesenteric), spleen, and skin epidermis was similar between WT and Gnaq−/− mice. Likewise, the numbers of T cells present in these tissues were indistinguishable between WT and Gnaq−/− bone marrow chimeras (Table I). Finally, we did not observe any reductions in the numbers of DCs present in the LNs and spleens of the Gnaq−/− mice (Table I). Indeed, if anything, some DC populations were elevated in the spleen and mesenteric LNs of the Gnaq−/− mice. Therefore, these data indicate that Gαq...
is not required for the migration of DCs or T cells to secondary lymphoid organs under homeostatic conditions.

Next, to test whether $G\alpha_i$ regulates DC migration to LNs in response to inflammatory stimuli, we sensitized the skin of WT and $Gnaq^{-/-}$ mice with FITC in acetone/dibutyl phthalate and prepared frozen tissue sections of the draining LNs 18 h after sensitization. The sections were stained with CD11c and CD90.2 to identify DCs within the T cell zone of the LNs. As shown in Fig. 6 A, the FITC-bearing DCs were easily detected in the T zone of WT LNs but were largely absent from the T cell area of $Gnaq^{-/-}$ LNs (Fig. 6 B). To determine whether this was caused by the inappropriate localization of the $Gnaq^{-/-}$ DCs to other regions within the LN, we used FACS analysis to enumerate the total number of FITC$^+$CD11c$^+$ClassII$^+$ DCs present in the draining LNs of the FITC-sensitized WT and $Gnaq^{-/-}$ mice. The number of FITC$^+$ DCs in the draining LNs of the FITC-sensitized WT and $Gnaq^{-/-}$ mice was reduced by 90% (Fig. 6 C), indicating that the DCs present in the skin of $Gnaq^{-/-}$ mice did not accumulate in secondary lymphoid tissues in response to inflammatory challenge. This was not caused by a deficit of DCs in the epidermis of $Gnaq^{-/-}$ mice, as the cells were present in equal numbers in the epidermis before stimulation (Table I). Nor did it appear that the $Gnaq^{-/-}$ DCs were unable to mature in response to inflammatory stimuli, as bone marrow–derived $Gnaq^{-/-}$ DCs matured equivalently to WT DCs in vitro in response to TNF-$\alpha$ (unpublished data). Thus, the data strongly suggest that $Gnaq^{-/-}$ DCs are unable to migrate to LNs in response to inflammatory challenge.
We previously demonstrated that CD38 and cADPR regulate the trafficking of CCR2-expressing monocytes both in vitro and in vivo (29). Given that CD38 is expressed on monocytes (not depicted) and that CD38 and Goq each regulate the migration of mature DCs to draining LNs in response to inflammatory stimuli (Fig. 6 A) (29), we hypothesized that Goq would also be required for the migration of CCR2+ monocytes to the inflamed skin. To test this hypothesis, we took advantage of a previously described model to monitor the migration of monocytes to inflamed skin (41). In brief, we lethally irradiated WT CD45.1+ B6 mice and reconstituted the mice with congenic CD45.2+ WT mice or blood-derived monocytes to the inflamed skin (41). As previously reported for this model (41), the resident Langerhans cells (LCs; CD11c+ ClassII+) in the skin were radiation resistant. Thus, the vast majority of LCs in the skin of both groups of chimeras were of host origin and expressed the CD11c+ (Fig. 6 E, day 0). In contrast, the hematopoietic cells isolated from all other sites (i.e., bone marrow, blood, and secondary lymphoid tissues) expressed the CD11c+ (unpublished data), indicating that these populations were radiation sensitive and had been replaced by cells derived from the donor (either WT or Goq−/−) bone marrow.

Next, we treated the epidermis of the chimeric animals with the inflammatory agent dinitrofluorobenzene (DNFB), as we previously showed that this contact stimulant activates LCs to migrate to LNs and induces the subsequent influx of bone marrow- or blood-derived monocytes to the inflamed epidermis, where the cells up-regulate MHCII and CD11c (29). 4 d after DNFB exposure, we isolated cells from the inflamed epidermis and again stained the cells with antibodies to CD45.1, CD45.2, CD11c, and MHCII I-Aq. As expected (29), DNFB exposure promoted the egress of the host-derived resident WT CD45.1+ LCs from the skin, resulting in a large decrease in this population in the epidermis of both WT and Goq−/− chimeras (Fig. 6 E, day 4). In the WT chimeras, the epidermis was largely repopulated with CD11c+ ClassII+ cells that expressed the CD11c+ (unpublished data), indicating that WT donor-derived monocytes were recruited to the inflamed skin. In striking contrast, the epidermis of the Goq−/− chimeras was very deficient in CD11c+ ClassII+ cells, and only a small number of CD45.2+ expressing (Goq−/−) CD11c+ ClassII+ cells were detected (Fig. 6 E, day 4). These data therefore indicate that Goq−/− monocytes were not efficiently recruited to the inflamed epidermis, despite the fact that the inflamed skin and initial resident LCs were of WT origin. Collectively, these data show that the alternative Goq-dependent chemo-kine receptor signaling pathway is critical for the in vivo trafficking of DCs and monocytes in response to inflammatory signals and indicates that this signaling pathway regulates innate immune responses and the cellular processes associated with inflammation.

**Table 1. Lymphoid and DC subsets in Goq−/− tissues**

| Populations* | Spleen | Axillary LN | Mesenteric LN | Epidermis |
|--------------|--------|------------|-------------|-----------|
|              | WT | Gnaq−/− | WT | Gnaq−/− | WT | Gnaq−/− | WT | Gnaq−/− |
| Total cells (×10⁶) | 102.2 ± 2.81 | 158.6 ± 9.8* | 7.5 ± 0.5 | 6.5 ± 0.5 | 13.3 ± 0.5 | 18 ± 0.7* | ND | ND |
| B cells (×10⁶) (CD19+) | 594.2 ± 67.4 | 788.6 ± 50.5* | 22.8 ± 4.7 | 20.8 ± 3 | 65.6 ± 3.9 | 89.2 ± 12.7 | ND | ND |
| T cells (×10⁶) (CD3+) | 243.4 ± 53.8 | 258.9 ± 21.1 | 18.3 ± 1.6 | 17.9 ± 2.3 | 35.4 ± 1.2 | 33.6 ± 7.6 | ND | ND |
| CD4+ T (×10⁶) (CD3+CD4+) | 200.4 ± 36.6 | 226.6 ± 17.7 | 15.9 ± 1.4 | 14.9 ± 2 | 30.6 ± 1.2 | 28.9 ± 6 | ND | ND |
| CD8+ T (×10⁶) (CD3+CD8+) | 37.9 ± 16.1 | 27.8 ± 3.7 | 2.2 ± 0.3 | 2.7 ± 0.3 | 4.4 ± 0.3 | 4.4 ± 1.5 | ND | ND |
| DCs (×10⁴) (CD11c+) | 525.9 ± 37.9 | 923.1 ± 95.7* | 23.1 ± 4.7 | 24.3 ± 4 | 87.6 ± 3.7 | 146.1 ± 26.2* | ND | ND |
| Myeloid DCs (CD14+CD11b+CD86−) (CD11c+CD11b+CD86−) | 159.4 ± 13.8 | 230.1 ± 35.8 | 16.5 ± 4 | 16.5 ± 3.5 | 20.1 ± 1 | 39.2 ± 4.7* | ND | ND |
| Lymphoid DCs (CD11c+CD11b+CD86−) (CD11c+CD11b+CD86−) | 41.7 ± 3.7 | 81.3 ± 15.1* | 0.8 ± 0.1 | 1 ± 0.1 | 2.2 ± 0.2 | 3.7 ± 1 | ND | ND |
| Plasmacytoid DCs (CD11c+GR1+ B220+) (CD11c+GR1+ B220+) | 28.8 ± 7.7 | 27.7 ± 3.6 | 0.9 ± 0.2 | 0.8 ± 0.2 | 2.8 ± 0.2 | 3.9 ± 1.1 | ND | ND |
| LCs (CD11c+) (CD11c+ClassII+Langerin+) (CD11c+ClassII+Langerin+) | ND | ND | ND | ND | ND | ND | 13.7 ± 2.9 | 13 ± 1.8 |

*Cells were isolated from various WT and Gnaq−/− lymphoid tissues (n = 5 mice per group), counted, and stained with the antibodies indicated, and the total number of each leukocyte population was determined. The data are presented as the mean ± SEM of each group. *, P < 0.05 as determined by an unpaired Student’s t test analysis. ND, not done.
model (1) correctly postulates a central role for Goi2 in leukocyte chemotaxis.

Despite the critical importance of Gi in chemokine-induced cell trafficking, it has been known for many years that chemokine receptors can also couple to other G proteins (for review see reference 44), including Gq family members (35, 45-47). Emerging data have revealed that these additional classes of G proteins regulate chemokine receptor-mediated functions such as IP3 generation and calcium release (35, 47-49), activation of NF-κB (50, 51), activation of tyrosine kinases (52, 53), receptor internalization (54, 55), and exocytosis (36). However, there was only sporadic evidence (56–59) to suggest that these other G proteins might also regulate the chemotactic response of the chemoattractant-stimulated leukocytes. Thus, the experiments presented in this paper are novel in that they reveal that Gi, although necessary, is not sufficient to induce chemotaxis of primary leukocytes to a large

**DISCUSSION**

Immune responses are coordinated by the migration of leukocytes between the blood, secondary lymphoid organs, and inflamed tissues (42). This well-orchestrated migration of hematopoietic cells is complex, but it is clear that chemokine receptors and the signaling molecules that reside downstream of these receptors are exceedingly important (43). Indeed, in vitro experiments using chemokine receptor–transfected cell lines revealed that chemokine receptors couple to Gi-containing G proteins and that activation of Gi, with the subsequent release of free βγ subunits, is required for chemotaxis (6). Likewise, experiments using the Gi inhibitor PTx (for review see reference 17) and our own experiments using Goi2-deficient cells (Table II) demonstrate that Goi-containing G proteins are crucial for the migration of primary hematopoietic cells to most of the known chemoattractants. Thus, the generally accepted classical chemokine receptor signaling
Finally, the choice of cell type examined is critical. For example, many previous biochemical studies used transfected cell lines (i.e., HEK293 and COS-7 cells) that do not express CD38 (unpublished data), which is an important component of the alternative signaling pathway (Table II) (28-30). In addition, in primary T cells and B cells, chemokine receptors such as CXCR4 and CCR7 signal via the classical pathway, but in DCs these same receptors signal via the alternative pathway (Table II). Thus, the alternative signaling pathway is difficult to visualize unless the correct primary cells are used, and these cells then need to be interrogated with the appropriate chemokines and functional analyses.

In addition to demonstrating that \( \text{G}_q^i \) is required for leukocyte chemotaxis to a subset of different chemokines, we also found that \( \text{G}_q^i \) plays a previously unappreciated role in regulating calcium mobilization after chemokine receptor ligation. It is well known that four of the PLC\( \beta \) isoforms can be directly activated by active GTP-bound \( \text{G}_q^q \), and that activation of these PLC\( \beta \) isoforms by \( \text{G}_q^q \) leads to IP\(_3\) generation and diacylglycerol production (64). However, it appears that the IP\(_3\) response in fMLF- or IL-8–stimulated primary mouse bone marrow neutrophils is mediated largely, if not exclusively, by \( \text{G}_q^i2 \)-containing G proteins, presumably through free \( \text{G}_q^i2 \)-mediated activation of one or more PLC\( \beta \) isoforms. In fact, \( \text{G}_q^q \) is not obligatorily required for the chemokine-induced early IP\(_3\)-dependent calcium response of neutrophils activated with either IL-8 (a ligand of the classical pathway) or fMLF (a ligand of the alternative pathway). Instead, an additional, alternative \( \text{G}_q^q \)-coupled pathway must be engaged before primary neutrophils and DCs can migrate (Table II), and this second, alternative \( \text{G}_q^q \)-dependent pathway is critically important for cell trafficking in vitro and, more importantly, in vivo, at least in response to inflammatory stimuli.

Although our data clearly document the existence of a second, alternative \( \text{G}_q^q \)-dependent chemokine receptor signaling pathway, the pathway appears to have been largely overlooked in previous chemokine receptor signaling studies. This was initially surprising to us, particularly given that \( \text{G}_q^q \) is widely expressed in myeloid cells and DCs (60, 61) and that there is strong evidence that other Gq family members, such as \( \text{G}_q^15/16 \), can mediate chemokine-induced IP\(_3\) induction (45, 49, 62). However, there are several reasons that can explain the apparent discrepancy between the older studies and the current study. First, given the critical importance of Gi in both the classical and alternative chemokine receptor signaling pathways, it is not possible to visualize the alternative signaling pathway unless \( \text{G}_q^q \) is selectively targeted in a \( \text{G}_q^i2 \)-sufficient cell and, to our knowledge, only a single study examining \( \text{G}_q^q \)-deficient leukocytes has been published (63). Second, many of the previous biochemical studies analyzed signaling through CXCR1/CXCR2, the receptors for IL-8 and Mip2 (5-7, 48). As we now know, these receptors are activated via the classical pathway, at least in bone marrow neutrophils. Third, although the alternative signaling pathway regulates chemotaxis, it does not modulate all chemokine receptor–induced activities (i.e., chemokinesis) (28), and studies that examined an inappropriate functional readout would not be expected to identify the alternative pathway.

Finally, the choice of cell type examined is critical. For example, many previous biochemical studies used transfected cell lines (i.e., HEK293 and COS-7 cells) that do not express CD38 (unpublished data), which is an important component of the alternative signaling pathway (Table II) (28-30). In addition, in primary T cells and B cells, chemokine receptors such as CXCR4 and CCR7 signal via the classical pathway, but in DCs these same receptors signal via the alternative pathway (Table II). Thus, the alternative signaling pathway is difficult to visualize unless the correct primary cells are used, and these cells then need to be interrogated with the appropriate chemokines and functional analyses.

In addition to demonstrating that \( \text{G}_q^q \) is required for leukocyte chemotaxis to a subset of different chemokines, we also found that \( \text{G}_q^q \) plays a previously unappreciated role in regulating calcium mobilization after chemokine receptor ligation. It is well known that four of the PLC\( \beta \) isoforms can be directly activated by active GTP-bound \( \text{G}_q^q \), and that activation of these PLC\( \beta \) isoforms by \( \text{G}_q^q \) leads to IP\(_3\) generation and diacylglycerol production (64). However, it appears that the IP\(_3\) response in fMLF- or IL-8–stimulated primary mouse bone marrow neutrophils is mediated largely, if not exclusively, by \( \text{G}_q^i2 \)-containing G proteins, presumably through free \( \text{G}_q^i2 \)-mediated activation of one or more PLC\( \beta \) isoforms. In fact, \( \text{G}_q^q \) is not obligatorily required for the chemokine-induced early IP\(_3\)-dependent calcium response of neutrophils activated with either IL-8 (a ligand of the classical pathway) or fMLF (a ligand of the alternative pathway). Instead, \( \text{G}_q^q \) appears to play a critical role in activating calcium influx through a plasma membrane channel. Although we do not yet know the identity of the \( \text{G}_q^q \)-activated calcium/cation

### Table II. Signaling requirements for chemotaxis

| Receptor | Cell type (mouse) | \( \text{G}_q^i2 \) dependent | \( \text{G}_q^q \) dependent | CD38 dependent | Classical or alternative pathway |
|----------|-------------------|-------------------------------|-------------------------------|----------------|-------------------------------|
| CCR1     | BM neutrophil     | Y                             | Y                             | Y              | Alternative                   |
| CCR2     | Immature DC       | Y                             | ?                             | Y              | Alternative?                  |
| CCR7     | DC                | Y                             | Y                             | Y              | Alternative                   |
| CCR7     | T cell            | Y                             | N                             | N              | Classical                     |
| CXCR1/2  | BM neutrophil     | Y                             | Y                             | Y              | Alternative                   |
| CXCR4    | DC                | Y                             | Y                             | Y              | Alternative                   |
| CXCR4    | T cell            | Y                             | N                             | N              | Classical                     |
| CXCR4    | B cell            | Y                             | N                             | N              | Classical                     |
| CXCR5    | B cell            | Y                             | N                             | N              | Classical                     |
| mFPR1    | BM neutrophil     | Y                             | Y                             | Y              | Alternative                   |
| mFPR1    | Inflam. neutrophil| Y                             | N                             | N              | Classical                     |
| mFPR2    | BM neutrophil     | Y                             | Y                             | Y              | Alternative                   |

Inflam., inflammatory; N, no; Y, yes.
ACTIVATION PATHWAY

Calcium influx is required for the chemotaxis of mouse bone marrow neutrophils to FMLF (28) and is also necessary for the chemotaxis of DCs to CCL19, CCL21, and CXCL12 (29). Although we cannot exclude the possibility that Goq regulates neutrophil chemotaxis by calcium-independent mechanisms, our data strongly suggest that a major mechanism by which Goq regulates chemotaxis is by controlling the sustained calcium entry response. Our data also show that Goq-mediated calcium influx in response to FMLF stimulation is dependent on an initial intracellular calcium release controlled by Goq. These results suggest that IP3, generated by Gi-activated PLCβ, sparks the global calcium response and, therefore, must be needed for calcium-dependent chemotaxis. However, published experiments showed that even when IP3 induction was completely abrogated in chemokine-stimulated neutrophils from PLCβ2 and PLCβ3 double-deficient mice, the chemotactic response of these PLCβ-deficient cells remained intact (10). Thus, these published experiments suggest that neither IP3-mediated intracellular calcium release nor calcium-dependent calcium influx are needed for neutrophil chemotaxis. Interestingly, the previously published experiments used “primed” PLCβ-deficient neutrophils isolated from inflammatory sites (10), and we used bone marrow “naïve” neutrophils in all of our experiments. As shown in Table II, neutrophils isolated from inflammatory sites respond to FMLF via the classical pathway and do not require CD38 or calcium influx for chemotaxis, whereas neutrophils isolated from the bone marrow of unmanipulated normal mice respond to FMLF via the alternative pathway. Again, it appears that context is critical when examining chemotaxis, and that the requirements for calcium mobilization (from either intracellular or extracellular stores) will vary depending on many factors, including cell type, the activation state of the cell, and which chemokine receptors are examined.

Although the discovery of the Goq-dependent alternative chemokine receptor signaling pathway begins to delineate some of the complexity of chemokine receptor signal transduction, it also leaves a whole range of new questions that will need to be addressed. For example, it is unclear why calcium mobilization is required for the chemotaxis of cells activated by the alternative chemokine receptor signaling pathway but is not required for cells activated via the classical pathway. We hypothesize that this is likely an issue of signaling thresholds and that in certain cells, synergy between the Gi– and Gq-coupled pathways is needed to optimally activate key downstream players involved in cytoskeletal rearrangements. Indeed, there is emerging data demonstrating that simultaneous activation of the Gi and Gq pathways can lead to enhanced calcium responses (65), but to date the physiologic relevance of this finding has not been appreciated. We believe that chemotaxis represents one out of perhaps multiple examples of physiologic functions that make use of this synergy.

We are also left with the questions of why some receptors like CXCR4 and CCR7 signal via the classical pathway in one cell type and via the alternative pathway in another cell type, and why mFPR1 signals via the classical pathway in inflammatory neutrophils and via the alternative pathway in bone marrow neutrophils. It is possible that there are simply more alternative pathways that are yet to be discovered and that these alternative pathways are also masked when the master regulator Gi is missing or inhibited. Indeed, there is data to suggest that the G12/G13 class of G proteins can also play a role in regulating the trafficking of some lymphocyte populations (66–68), and in HL-60 cells, G12/G13 was demonstrated to play a crucial role in cell polarity (69). Thus, it is likely that chemokine receptor signaling will be found to be even more heterogeneous, and that the simple generic chemokine receptor signaling model will need to be abandoned in favor of more complex models that illustrate how simple modifications of the signal transduction circuitry can generate different downstream physiologic consequences.

Finally, regardless of whether there are more alternative chemokine receptor signaling pathways awaiting discovery, our data illuminate a novel Goq-coupled signaling pathway that regulates chemokine receptor signal transduction and chemotaxis. Most importantly, our data show that this novel Goq-dependent pathway plays a critical and nonredundant role in cell migration. However, although the Goq-dependent chemokine receptor signaling pathway is required for the migration of DCs and monocytes in vivo in response to inflammatory stimuli, it does not appear to be required for the in vivo immigration of leukocytes to secondary lymphoid organs and peripheral tissues under homeostatic conditions. Thus, further study of the Goq-coupled chemokine receptor signaling pathway should allow us to identify other additional key signaling molecules that may be targets of drugs that can be used to inhibit chronic inflammation.

MATERIALS AND METHODS

Mice and bone marrow reconstitutions. C57BL/6j (B6), CD45.1 congenic B6, C57BL/6j 129 Gqα5-/- (N12 backcross to C57BL/6j) (28), Gnaq-/- (N>5 backcross to C57BL/6j) (70) mice were bred and maintained in the Trudeau Institute breeding facility. Gnaq-/- mice are born runted and often do not live to adulthood (71). Therefore, all in vitro chemotaxis and calcium signaling assays were routinely performed using cells isolated from lethally irradiated B6 recipients that were reconstituted with B6, Gnaq-/-, or Gnaq2-/- bone marrow 8 wk previously. This approach allowed us to examine the impact of Gnaq protein deficiency specifically within hematopoietic lineage cells and permitted us to obtain cells from larger numbers of adult animals for the analyses. Bone marrow chimeric mice were generated by reconstituting lethally irradiated...
recipients (950 cGy from a 137Cs source) with 10^7 total bone marrow cells isolated from appropriate donor mice. Mice were allowed to reconstitute for 8 wk before experiments. The Trudeau Institute Institutional Animal Care and Use Committee approved all procedures involving animals.

Reagents. Reagents were obtained from R&D Systems (CXCL12, CCL3, and CCL19), Sigma-Aldrich (PAF, IL-8, MLF, PTx, and DNFb), Calbiochem (2-APB), and BD Biosystems (all antibodies). 8Br-cADPR (provided by T. Waltho, University of Minnesota, Minneapolis, MN) was prepared as previously described (72). All reagents were used at the concentrations indicated in the text and figure legends.

Cell subset quantification, cell purification, and chemotaxis assays. Cells were isolated from the spleen, LNs (axillary and mesenteric), and skin epidermis of either WT or Gnaq^-/- chimeric mice. The total number of viable cells was determined, the cells were stained with fluorochrome-labeled antibodies, and the total number of B cells (CD19^+), T cells (CD3^+ and CD4^+ or CD8^+), myeloid DCs (CD11c^+ CD11b^-CD86^-), lymphoid DCs (CD11c^+ CD11b^-CD86^+), plasmacytoid DCs (CD11c^-GR1^-B220^-), and LCs (CD11c^-CD1^-Ab^-Langerin^-) was determined.

To purify bone marrow neutrophils, bone marrow cells were first stained with biotinylated anti-GR1 antibody and MACS streptavidin microbeads (Miltenyi Biotech) and then positively selected on a MACS midi column. CD4 T cells were purified using CD4 microbeads (Miltenyi Biotec) and then positively selected on a MACS midi column. CD4 T cells were purified using CD4 microbeads (Miltenyi Biotech). To isolate DCs, single-cell suspensions were prepared from pooled spleens and mesenteric, inguinal, and axillary LNs that were perfused with collagenase D and incubated for 60 min at 37°C. The cells were purified by two rounds of positive selection using biotinylated anti-CD11c antibody and streptavidin microbeads. The purity of all cell populations was routinely >90%. In vitro chemotaxis assays were performed using 24-well transwell plates (Corning) with either a 3-µm (for neutrophils) or 5-µm (DCs and T cells) pore size polycarbonate filter. In some experiments, the isolated cells were first pretreated for 20 min with 100 µM 8Br-cADPR. After any pretreatments, the cells were added to the top chamber of the transwell (10^5 DCs and 10^6 T cells or neutrophils per well, respectively). Transwell plates were incubated for 45 min (neutrophils) or 2 h (DCs and T cells). The transmigrated cells were collected from the lower chamber, fixed, and counted on a flow cytometer. The absolute number of cells in each sample was determined by spiking each sample with a known number of 20-µm fluorescent microbeads that were simultaneously counted on the flow cytometer. In some cases, the results are expressed as the mean ± SD of the chemotactic index (CI). The CI represents the fold increase in the number of migrated cells in response to chemotactic attractants over the spontaneous cell migration (to control medium).

Calcium mobilization assays. 10^6 bone marrow neutrophils and 10^6 DCs per milliliter were loaded with a mixture of Fluo-3 AM and Fura-red AM, as previously described (28, 29). In some experiments, the cells were preincubated for 20 min in 8Br-cADPR or 2-APB. In other experiments, cells were pretreated with 500 ng/ml PTx for 4 h, washed, and stimulated with chemokines. The accumulation of intracellular free calcium was assessed by flow cytometry by measuring the fluorescence emission of Fluo-3 in the FL-1 channel and Fura-red in the FL-3 channel over time. Data were analyzed using Flowjo software (version 3.0; TreeStar, Inc.). Relative intracellular free calcium levels are expressed as the ratio between Fluo-3 and Fura-red fluorescence intensity.

In vivo DC activation and migration assays. To measure the migration of monocytes to the skin, we followed the protocol described by Merad et al. (41). In brief, CD45.1^- B6 recipient mice were lethally irradiated and reconstituted with either CD45.2^- B6 bone marrow or with CD45.2^- Gnaq^-/- bone marrow. 8 wk after reconstitution, cells from skin epidermal sheets were isolated (73) and stained with antibodies to CD45.1, CD45.2, CD11c, and ClassI to determine whether the LCs (CD11c^-ClassII^+) were of donor (CD45.2^+) or host (CD45.1^+) origin. The remaining mice were then exposed to DNFB (0.5% applied to the ear). On day 4 after DNFB application, cells were isolated from skin epidermal sheets and stained with the same panel of antibodies. The number of donor (CD45.2^+) and host-derived (CD45.1^-) LCs (CD11c^-ClassII^+) present in the skin samples was then determined by cell counting and FACS.

To track DC migration from inflamed skin to LNs, 25 µl FITC (8 mg/ml in 1:1 acetone/dibutylphthalate) was applied to two shaved abdominal areas of B6 or Gnaq^-/- (nonchimeric) animals. Inguinal LNs were removed after 18–24 h, counted, stained with antibodies to class II and CD11c, and analyzed by flow cytometry. The total number of migrated FITC^-/CD11c^- classII^- DCs was determined.

Immunohistology. 5-µm frozen sections were prepared from inguinal LNs isolated from FITC^-/sensitized mice at 18 h after exposure. Sections were stained with antibodies to CD11c (conjugated to Alexa Fluor 594; Invitrogen) and CD90.2 (conjugated to Alexa Fluor 350; Invitrogen). Slides were viewed at 200× magnification using a microscope (AxioPhot 2; Carl Zeiss MicroImaging, Inc.), and images were captured with a digital camera (AxioCam; Carl Zeiss MicroImaging, Inc.) using Axiovision software (version 3.0; Carl Zeiss MicroImaging, Inc.). Images were cropped and scaled using Canvas software (version 9.0; ACD Systems).

Statistical analysis. Datasets were analyzed using Prism software (version 4.0 for Macintosh; GraphPad Software). Student’s t tests were applied to the datasets, and P < 0.05 was considered statistically significant. All data are shown as the mean ± SD.

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