

# Identification of an alternative $G\alpha_q$ -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  $G\alpha_{i2}$ -containing  $G_i$  proteins. However, unlike the classical pathway, the alternative pathway is also dependent on the  $G_q$  class of G proteins. We show that  $G\alpha_q$ -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,  $G\alpha_q$ -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  $G\alpha_q$  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.**

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Abbreviations used: 2-APB, 2-aminoethoxydiphenylborate; ADPR, adenosine diphosphoribose; cADPR, cyclic ADPR; CCL and CCR, CC chemokine ligand and receptor, respectively; CI, chemotactic index; CXCL and CXCR, CXC chemokine ligand and receptor, respectively; DNFB, dinitrofluorobenzene; fMLF, *N*-formyl methionyl leucyl phenylalanine; IP<sub>3</sub>, inositol trisphosphate; LC, Langerhans cell; mFPR, mouse formyl peptide receptor; NAD<sup>+</sup>, nicotinamide adenine dinucleotide; PAF, platelet-activating factor; PLC, phospholipase; PTx, pertussis toxin.

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 G protein complex that directly associates with chemokine receptors and transduces signals from these receptors to other key intracellular signaling molecules (2–5). The G proteins associated with chemokine receptors contains three subunits:  $G\alpha_i$ ,  $\beta$ , and  $\gamma$ . The activation of G proteins is induced by the

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binding of GTP to  $G\alpha_i$  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 that control cytoskeletal changes necessary for chemotaxis (8–10). In addition, the  $\beta\gamma$  subunits released from chemokine receptor-associated G proteins are capable of activating phospholipase (PLC)  $\beta_2$  and PLC $\beta_3$  (11, 12), the enzymes responsible for inositol trisphosphate (IP<sub>3</sub>) production and intracellular calcium release from IP<sub>3</sub>-gated stores (13). Inhibitors of  $G_i$ , 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

(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<sup>+</sup> and NADP<sup>+</sup> (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 signals has important immunological consequences, as mice deficient in CD38 (*Cd38*<sup>-/-</sup>) 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 $\alpha_i$ -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 $\alpha_i$ . 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*<sup>-/-</sup>) 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 mouse 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*<sup>-/-</sup> neutrophils migrate in transwell chambers in response to an optimal concentration of fMLF (Fig. 1 A). In contrast, chemotaxis of *Cd38*<sup>-/-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> 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 $\alpha_q$

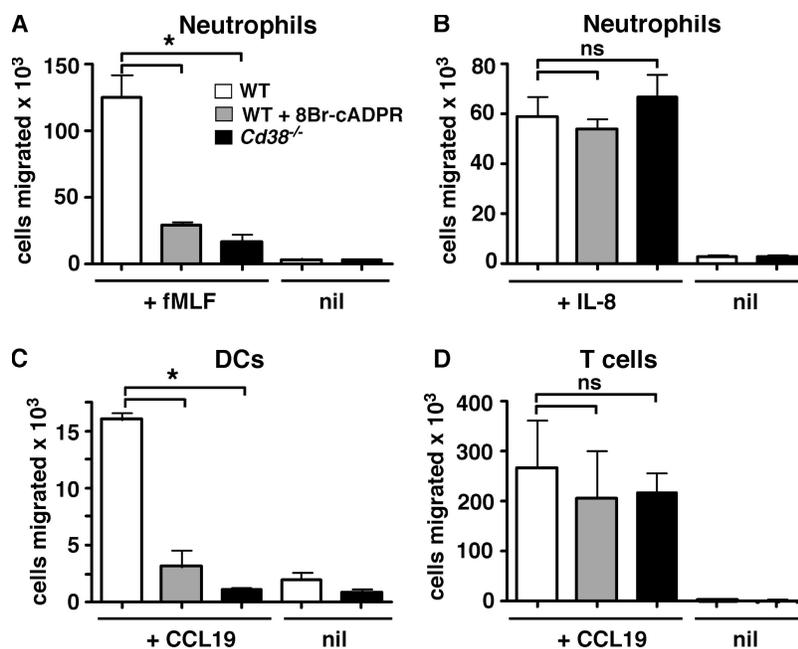
Our data suggested that there was considerably more diversity or heterogeneity in the molecular signals that regulate chemotaxis than previously appreciated. To better understand the diversity between chemokine receptors, we examined the response of WT and *Cd38*<sup>-/-</sup> neutrophils to platelet-activating 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 $\alpha_i$  (34–36). Therefore, we loaded bone marrow WT and *Cd38*<sup>-/-</sup> 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

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*<sup>-/-</sup> 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*<sup>-/-</sup> DCs (unpublished data). Thus, calcium signaling through the PAFR appears to be regulated by CD38 and cADPR.

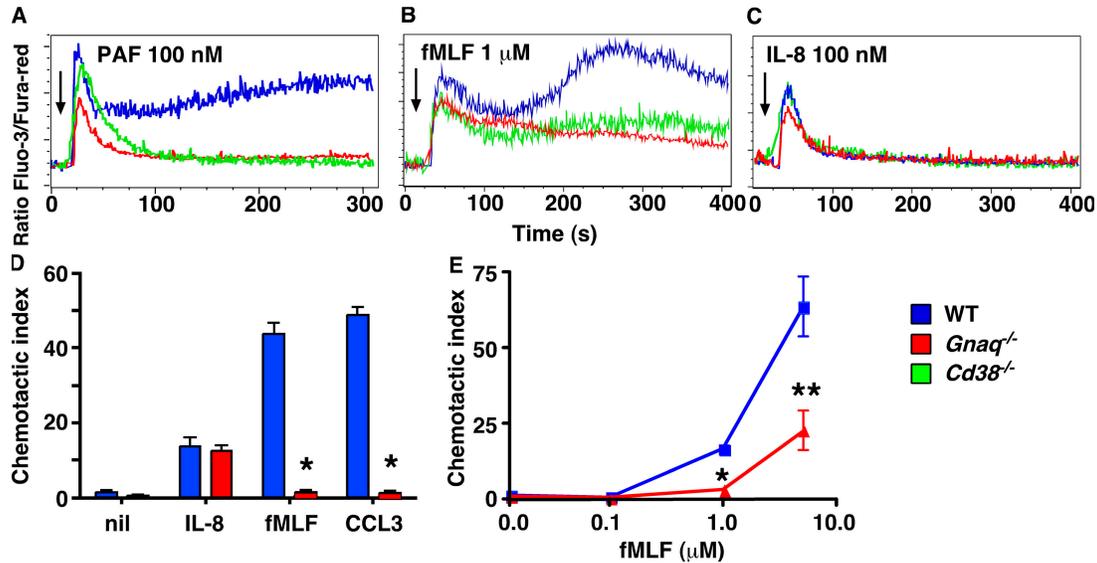
It has been previously demonstrated that PTx-treated PAF-activated cells produce IP<sub>3</sub> (34–36), indicating that the PAFR must couple to one or more members of the Gq family of G proteins that are capable of activating PLCβ and inducing IP<sub>3</sub> formation (32, 33). To assess whether the PAFR couples to Gα<sub>q</sub>, we purified bone marrow neutrophils from chimeric mice that lacked Gα<sub>q</sub> (*Gnaq*<sup>-/-</sup>) 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

human neutrophils (34), the immediate calcium release from IP<sub>3</sub>-gated intracellular stores was reduced, although not absent, in the PAF-stimulated *Gnaq*<sup>-/-</sup> neutrophils relative to WT neutrophils (Fig. 2 A). Furthermore, in a manner identical to what we observed with *Cd38*<sup>-/-</sup> neutrophils, the PAF-induced calcium response was not sustained in the *Gnaq*<sup>-/-</sup> neutrophils (Fig. 2 A), suggesting that Gα<sub>q</sub> regulates both intracellular calcium release and calcium influx in these cells.

These results suggested that CD38 and Gα<sub>q</sub> might both be involved in activating calcium influx in chemokine-stimulated cells. If this conclusion is correct, then we postulated that Gα<sub>q</sub>, 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*<sup>-/-</sup>, and *Gnaq*<sup>-/-</sup> 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*<sup>-/-</sup> neutrophils (Fig. 2 B). Interestingly, the calcium response of the fMLF-activated *Gnaq*<sup>-/-</sup> neutrophils was very similar to that of the *Cd38*<sup>-/-</sup> 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*<sup>-/-</sup> neutrophils during the second calcium influx phase of the response (28), suggesting a



**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*<sup>-/-</sup> 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<sup>+</sup> DCs and splenic CD4<sup>+</sup> T cells were purified from WT and *Cd38*<sup>-/-</sup> 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  $G\alpha_q$ . (A–C) Bone marrow neutrophils from WT (blue), *Cd38*<sup>-/-</sup> (green), and *Gnaq*<sup>-/-</sup> (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*<sup>-/-</sup> 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. \*,  $P \leq 0.0001$ ; or \*\*,  $P < 0.03$  between WT and *Gnaq*<sup>-/-</sup> neutrophils.

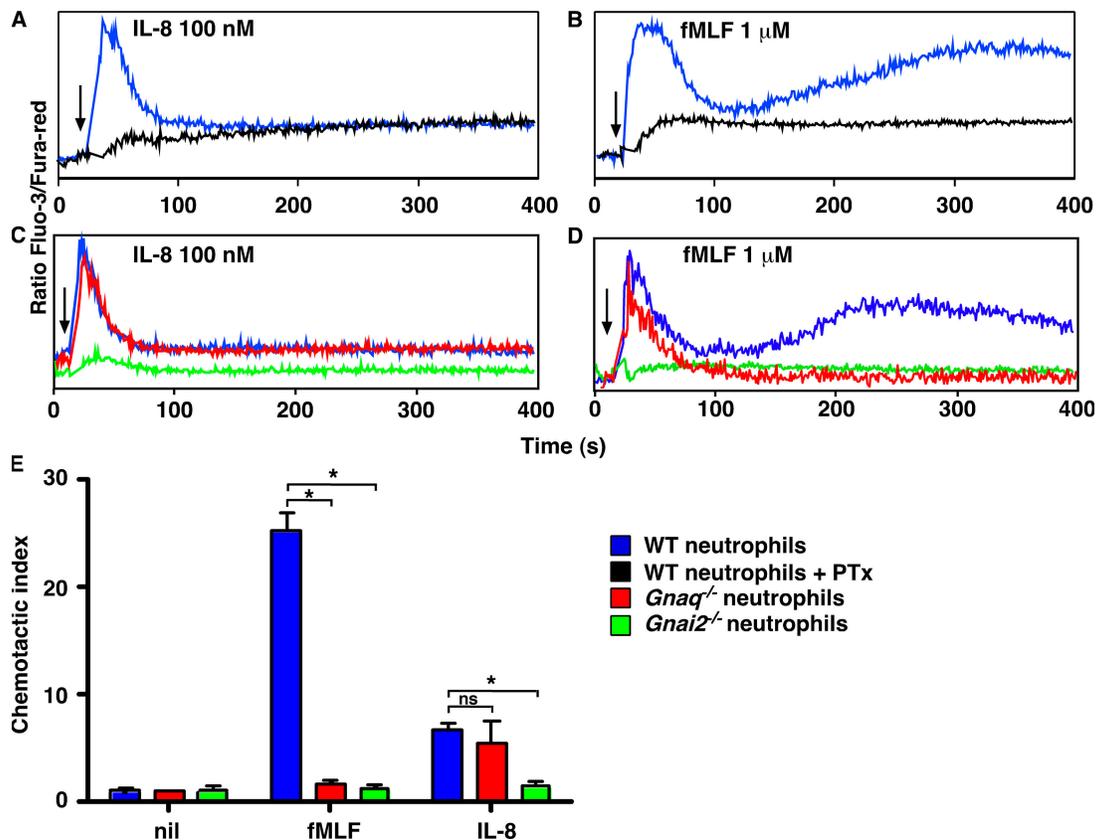
prominent role for  $G\alpha_q$  in regulating calcium entry rather than intracellular calcium release in this response. To bolster this conclusion, we next examined the calcium response of IL-8-activated *Gnaq*<sup>-/-</sup> neutrophils, as the calcium response to IL-8 in mouse bone marrow neutrophils is almost entirely caused by IP<sub>3</sub>-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  $G\alpha_q$  (Fig. 2 C), again very similar to what we observed with the *Cd38*<sup>-/-</sup> neutrophils (Fig. 2 C).

Collectively, these data suggested a strong positive correlation between CD38 and  $G\alpha_q$ , 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  $G\alpha_q$  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*<sup>-/-</sup> 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*<sup>-/-</sup> neutrophils to IL-8 was completely normal. However the *Gnaq*<sup>-/-</sup> 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*<sup>-/-</sup> 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  $G\alpha_q$ - 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 $G\alpha_i$ and $G\alpha_q$

The results showing that chemotaxis of neutrophils to mFPR1 and CCR1 ligands is  $G\alpha_q$  dependent were surprising, as the  $G_i$  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,  $G\alpha_{i2}$ -deficient (*Gnai2*<sup>-/-</sup>), and *Gnaq*<sup>-/-</sup> neutrophils that were activated with either IL-8 or fMLF. Similar to the PTx-treated cells, *Gnai2*<sup>-/-</sup> 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*<sup>-/-</sup> neutrophils to IL-8 was normal (Fig. 3 C), and the calcium response of the *Gnaq*<sup>-/-</sup> cells to fMLF was only partially impaired (Fig. 3 D). Not surprisingly, *Gnai2*<sup>-/-</sup> neutrophils were unresponsive to both IL-8 and fMLF in the in vitro chemotaxis assay (Fig. 3 E). In contrast, the chemotactic response of



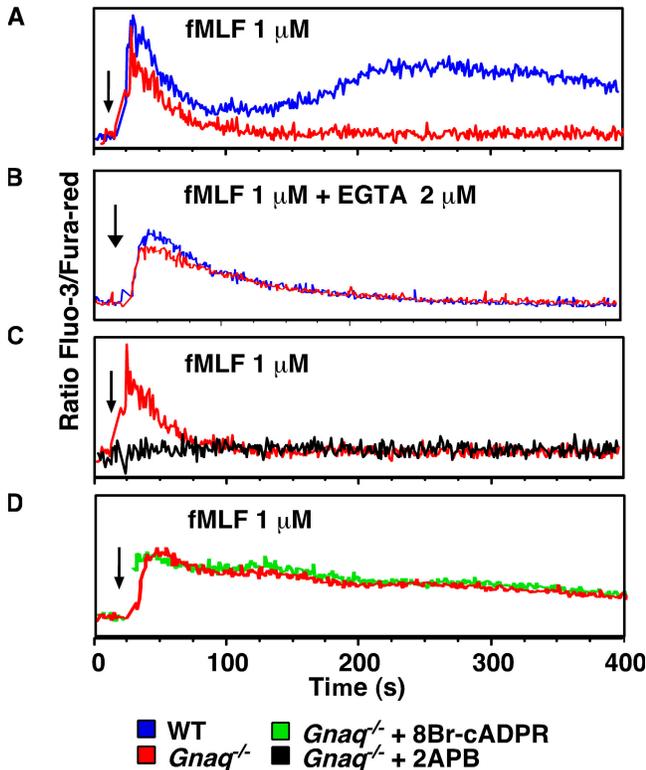
**Figure 3. Signaling through a subset of chemokine receptors is dependent on  $G\alpha_{12}$  and  $G\alpha_q$ .** (A–D) Bone marrow neutrophils from WT (blue or black), *Gnaq*<sup>-/-</sup> (red), or *Gnai2*<sup>-/-</sup> (green) mice were preincubated in media or 500 ng/ml PTx (black) for 4 h. The cells were loaded with Fluo-3 and Fura-red and stimulated with 100 nM IL-8 (A and C) or 1 μM fMLF (B and D). 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. (E) Bone marrow neutrophils from WT, *Gnaq*<sup>-/-</sup>, or *Gnai2*<sup>-/-</sup> mice were placed in transwells containing media (nil), 1 μM fMLF, or 100 nM IL-8. 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 of triplicate cultures. The data shown are representative of four or more independent experiments. \*,  $P \leq 0.0001$  between WT neutrophils and the indicated groups. ns, not significant.

*Gnaq*<sup>-/-</sup> neutrophils to fMLF was ablated, whereas the response to IL-8 was completely intact (Fig. 3 E). Collectively, the data indicate that some chemokine receptors expressed by mouse bone marrow neutrophils, such as CXCR1/2, couple to G proteins containing  $G\alpha_{12}$  but not  $G\alpha_q$ . However other receptors, such as mFPR1 and CCR1, couple to two different classes of G proteins,  $G\alpha_{12}$  and  $G\alpha_q$ .

#### $G\alpha_q$ regulates calcium influx in chemokine-stimulated neutrophils

The “signature” activity of the Gq class of G proteins is to activate one or more PLC $\beta$  isoforms, which catalyze production of IP<sub>3</sub> and promote intracellular calcium release (32, 33). This activity is performed by the  $\alpha$  subunit of the trimeric  $G\alpha_q$ -containing proteins (32, 33). Therefore, it was surprising to find that the largest alteration in the calcium response of the fMLF-stimulated *Gnaq*<sup>-/-</sup> neutrophils appeared during the later calcium entry phase of the response rather than during the initiation of the calcium response. To better understand how  $G\alpha_q$  regulates chemokine receptor signal transduction,

we examined the calcium response of chemokine-stimulated *Gnaq*<sup>-/-</sup> mouse bone marrow neutrophils in more detail. Initially, we stimulated *Gnaq*<sup>-/-</sup> neutrophils with fMLF in the presence or absence of EGTA (to chelate the extracellular calcium) so that we could assess the relative contribution of  $G\alpha_q$  to intracellular calcium release and extracellular calcium influx. Identical to our earlier results, the calcium response of fMLF-stimulated *Gnaq*<sup>-/-</sup> neutrophils initiated apparently normally but was not sustained and rapidly dropped to background levels (Fig. 4 A). Indeed, as shown in Fig. 4 B, the calcium response of the EGTA-treated fMLF-activated *Gnaq*<sup>-/-</sup> neutrophils was very similar to that seen in equivalently treated WT neutrophils, indicating that  $G\alpha_q$  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<sub>3</sub> dependent, we asked whether we could block the remaining calcium response in *Gnaq*<sup>-/-</sup> neutrophils by treating the fMLF-activated *Gnaq*<sup>-/-</sup> cells with the IP<sub>3</sub> inhibitor 2-aminoethoxydiphenylborate (2-APB). As shown in Fig. 4 C, the calcium



**Figure 4.**  $G\alpha_q$  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  $\mu$ M 8Br-cADPR (green), or 100  $\mu$ M 2-APB (black) for 20 min. The cells were loaded with Fluo-3 and Fura-red and stimulated with 1  $\mu$ M 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.

response was ablated in the 2-APB-treated  $Gnaq^{-/-}$  neutrophils, similar to that observed with fMLF-activated  $Gnai2^{-/-}$  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  $G\alpha_q$  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  $G\alpha_{i2}$  controls the  $IP_3$ -gated calcium release, and that  $G\alpha_q$  and CD38 coordinately sustain the calcium response by activating calcium entry in fMLF-activated mouse bone marrow neutrophils. The data further suggest that the  $G\alpha_{i2}$ -dependent calcium release is necessary for the initiation of the sustained calcium response in the fMLF-activated

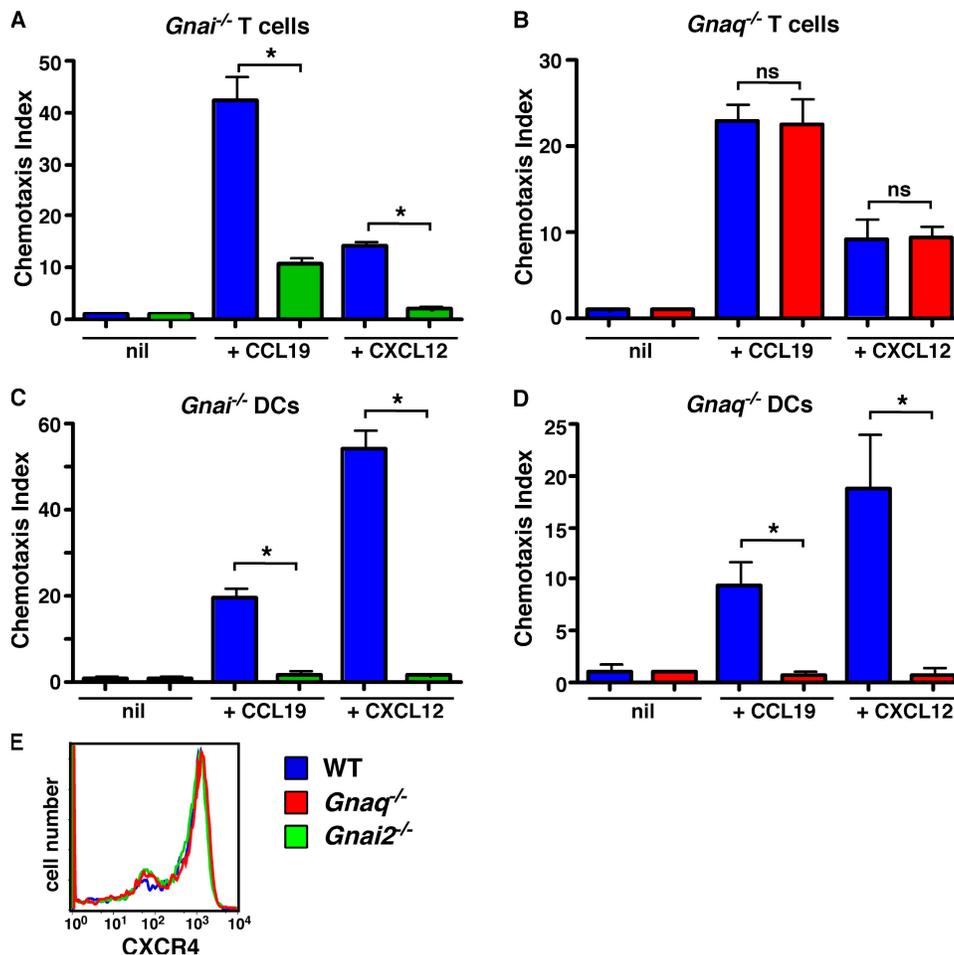
bone marrow neutrophils. However, it is also possible that  $G\alpha_{i2}$  additionally regulates the CD38- and  $G\alpha_q$ -dependent calcium influx response by other mechanisms unrelated to intracellular calcium release.

#### $G\alpha_q$ differentially regulates chemokine receptor signaling in DCs and T lymphocytes

Because  $G\alpha_q$  and CD38 are both required for productive mFPR1 and CCR1 signaling in mouse neutrophils, we postulated that  $G\alpha_q$  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  $G\alpha_q$  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  $G\alpha_{i2}$ . To test these predictions, we measured the in vitro chemotaxis of DCs and T cells from WT,  $Gnaq^{-/-}$ , and  $Gnai2^{-/-}$  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  $G\alpha_{i2}$  (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  $G\alpha_{i2}$ , but not  $G\alpha_q$ , 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,  $Gnai2^{-/-}$ , and  $Gnaq^{-/-}$  DCs (Fig. 5 E). Thus,  $G\alpha_q$ , 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  $G\alpha_q$ /CD38-dependent signaling pathway in one cell type but not necessarily in all cell types.

#### Inflammation-induced migration of DCs and monocytes requires $G\alpha_q$

Previous results showed that  $G\alpha_q$  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  $G\alpha_q$  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  $G\alpha_q$



**Figure 5.** DC migration to CCR7 and CXCR4 ligands is regulated by both  $G\alpha_q$  and  $G\alpha_{i2}$ , but only  $G\alpha_{i2}$  is necessary for the migration of T cells to the same ligands. (A and B) Spleen cells were isolated from WT (blue), *Gnai2*<sup>-/-</sup> (green), and *Gnaq*<sup>-/-</sup> (red) mice and placed in transwell chambers containing media (nil), 300 ng/ml CCL19, or 300 ng/ml CXCL12. The number of CD4<sup>+</sup> T cells placed in the top chamber and the number of CD4<sup>+</sup> T cells that migrated to the bottom chamber after 2 h were determined by FACS. The percentage of CD4<sup>+</sup> T cells that migrated in response to the chemokine gradient was calculated, and the CI was determined. The data are shown as the mean  $\pm$  SD of the CI of triplicate cultures. (C–E) CD11c<sup>+</sup> cells were purified from collagenase-digested spleens and LNs of WT, *Gnai2*<sup>-/-</sup>, and *Gnaq*<sup>-/-</sup> mice and placed in transwell chambers containing media (nil), 50 ng/ml CCL19, or 100 ng/ml CXCL12. After 2 h, the cells that migrated to the bottom chamber in response to the chemokine gradient were collected and enumerated by FACS. The results are expressed as the mean  $\pm$  SD of the CI of triplicate cultures. (E) CXCR4 expression levels on purified CD11c<sup>+</sup> cells were determined by FACS. \*,  $P \leq 0.004$  between WT cells and the indicated groups. The data shown are representative of at least three independent experiments. ns, not significant.

is not required for the migration of DCs or T cells to secondary lymphoid organs under homeostatic conditions.

Next, to test whether  $G\alpha_q$  regulates DC migration to LNs in response to inflammatory stimuli, we sensitized the skin of WT and *Gnaq*<sup>-/-</sup> 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*<sup>-/-</sup> LNs (Fig. 6 B). To determine whether this was caused by the inappropriate localization of the *Gnaq*<sup>-/-</sup> DCs to other regions within the LN, we used FACS analysis to enumerate the total number of FITC<sup>hi</sup>CD11c<sup>+</sup>ClassII<sup>+</sup> DCs

present in the draining LNs of the FITC-sensitized WT and *Gnaq*<sup>-/-</sup> mice. The number of FITC-labeled DCs present in the *Gnaq*<sup>-/-</sup> LN was reduced by >90% (Fig. 6 C), indicating that the DCs present in the skin of *Gnaq*<sup>-/-</sup> 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*<sup>-/-</sup> mice, as the cells were present in equal numbers in the epidermis before stimulation (Table I). Nor did it appear that the *Gnaq*<sup>-/-</sup> DCs were unable to mature in response to inflammatory stimuli, as bone marrow-derived *Gnaq*<sup>-/-</sup> DCs matured equivalently to WT DCs in vitro in response to TNF- $\alpha$  (unpublished data). Thus, the data strongly suggest that *Gnaq*<sup>-/-</sup> DCs are unable to migrate to LNs in response to inflammatory challenge.

**Table I.** Lymphoid and DC subsets in *Gnaq*<sup>-/-</sup> tissues

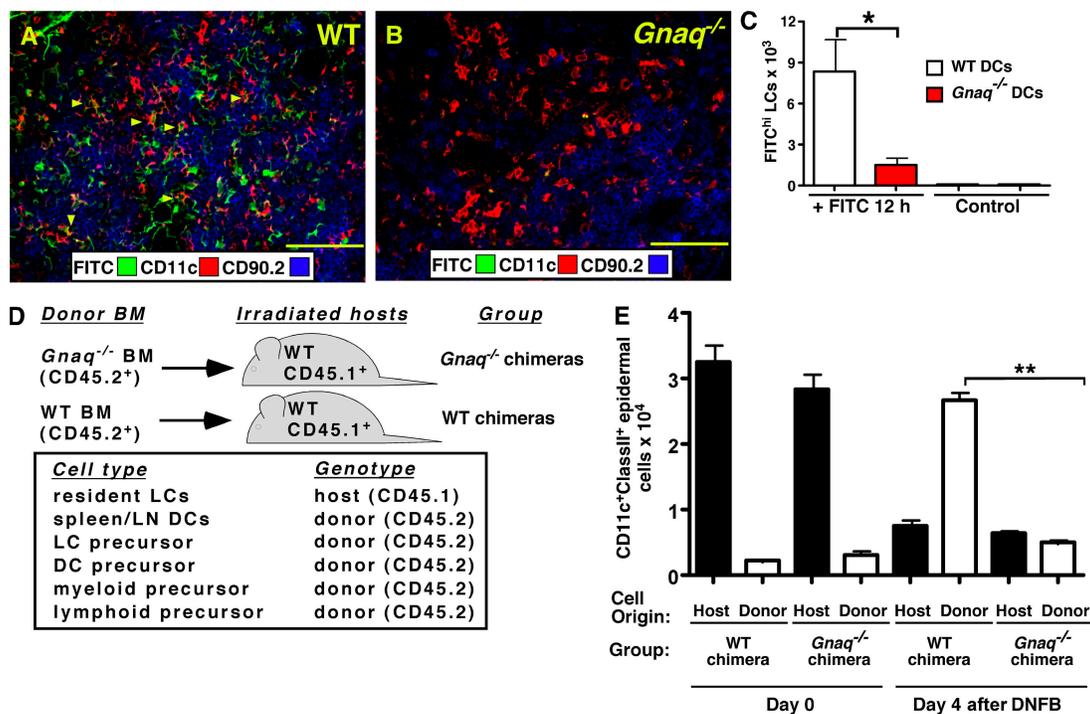
Populations <sup>a</sup>	Spleen		Axillary LN		Mesenteric LN		Epidermis	
	WT	<i>Gnaq</i> <sup>-/-</sup>	WT	<i>Gnaq</i> <sup>-/-</sup>	WT	<i>Gnaq</i> <sup>-/-</sup>	WT	<i>Gnaq</i> <sup>-/-</sup>
Total cells (×10 <sup>6</sup> )	102.2 ± 2.81	158.6 ± 9.8*	7.5 ± 0.5	6.5 ± 0.5	13.3 ± 0.5	18 ± 0.7*	ND	ND
B cells (×10 <sup>6</sup> ) (CD19 <sup>+</sup> )	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 (×10 <sup>6</sup> ) (CD3 <sup>+</sup> )	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 <sup>+</sup> T (×10 <sup>6</sup> ) (CD3 <sup>+</sup> CD4 <sup>+</sup> )	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 <sup>+</sup> T (×10 <sup>6</sup> ) (CD3 <sup>+</sup> CD8 <sup>+</sup> )	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 (×10 <sup>4</sup> ) (CD11c <sup>+</sup> )	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 (×10 <sup>4</sup> ) (CD11c <sup>+</sup> CD11b <sup>+</sup> CD8α <sup>-</sup> )	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 (×10 <sup>4</sup> ) (CD11c <sup>+</sup> CD11b <sup>-</sup> CD8α <sup>+</sup> )	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 (×10 <sup>4</sup> ) (CD11c <sup>int</sup> GR1 <sup>+</sup> B220 <sup>int</sup> )	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 (×10 <sup>3</sup> ) (CD11c <sup>+</sup> ClassII <sup>+</sup> Langerin <sup>+</sup> )	ND	ND	ND	ND	ND	ND	13.7 ± 2.9	13 ± 1.8

<sup>a</sup>Cells were isolated from various WT and *Gnaq*<sup>-/-</sup> 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.

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 Gα<sub>q</sub> each regulate the migration of mature DCs to draining LNs in response to inflammatory stimuli (Fig. 6 A) (29), we hypothesized that Gα<sub>q</sub> would also be required for the migration of CCR2<sup>+</sup> 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<sup>+</sup> B6 mice and reconstituted the mice with congenic CD45.2<sup>+</sup> WT (WT chimeras) or CD45.2<sup>+</sup> *Gnaq*<sup>-/-</sup> bone marrow (*Gnaq*<sup>-/-</sup> chimeras; Fig. 6 D). 8 wk after reconstitution, we isolated cells from skin epidermal sheets (isolated from the ear) of the WT and *Gnaq*<sup>-/-</sup> chimeras and stained the cells with antibodies to CD45.1, CD45.2, CD11c, and MHCII I-A<sup>b</sup>. As previously reported for this model (41), the resident Langerhans cells (LCs; CD11c<sup>+</sup>ClassII<sup>+</sup>) 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 CD45.1 allele (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 CD45.2 allele (unpublished data), indicating that these populations were radiation sensitive and had been replaced by cells derived from the donor (either WT or *Gnaq*<sup>-/-</sup>) 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-A<sup>b</sup>. As expected (29), DNFB exposure promoted the egress of the host-derived resident WT CD45.1<sup>+</sup> LCs from the skin, resulting in a large decrease in this population in the epidermis of both WT and *Gnaq*<sup>-/-</sup> chimeras (Fig. 6 E, day 4). In the WT chimeras, the epidermis was largely repopulated with CD11c<sup>+</sup>ClassII<sup>+</sup> cells that expressed the CD45.2 allele (Fig. 6 E, day 4), indicating that WT donor-derived monocytes were recruited to the inflamed skin. In striking contrast, the epidermis of the *Gnaq*<sup>-/-</sup> chimeras was very deficient in CD11c<sup>+</sup>ClassII<sup>+</sup> cells, and only a small number of CD45.2-expressing (*Gnaq*<sup>-/-</sup>) CD11c<sup>+</sup>ClassII<sup>+</sup> cells were detected (Fig. 6 E, day 4). These data therefore indicate that *Gnaq*<sup>-/-</sup> 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 Gα<sub>q</sub>-dependent chemokine 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.



**Figure 6. The in vivo migration of DCs and monocytes is dependent on  $G\alpha_q$ .** (A and B) The abdominal skin of WT (A) or *Gnaq*<sup>-/-</sup> (B) mice was shaved and painted with FITC. The draining inguinal LNs were removed 18 h after sensitization, and frozen sections were prepared for immunohistology. The sections were stained with anti-CD11c and anti-CD90.2 to identify DCs and T cells, respectively. FITC<sup>+</sup> cells and cells that were FITC<sup>+</sup> and expressed CD11c (orange cells, indicated with yellow arrowheads) were found primarily within the T cell zone of the LN. Bar, 100  $\mu$ m. (C) The inguinal LNs were isolated 20 h after sensitization with FITC, collagenase digested, counted, stained with antibodies to CD11c and MHCII I-Ab, and analyzed by FACS. The number of FITC<sup>hi</sup>CD11c<sup>+</sup>ClassII<sup>+</sup> DCs is indicated.  $n = 3$  mice per group. \*,  $P \leq 0.008$  between WT and *Gnaq*<sup>-/-</sup> mice. The data are representative of three independent experiments with at least three mice per group. (D) Lethally irradiated CD45.1<sup>+</sup> C57BL/6J hosts were reconstituted with either CD45.2<sup>+</sup> WT bone marrow (WT chimeras) or CD45.2<sup>+</sup> *Gnaq*<sup>-/-</sup> bone marrow (*Gnaq*<sup>-/-</sup> chimeras). Resident LCs are radiation resistant and remain of host origin (reference 41). All other hematopoietic cells, including DCs and monocytes, are replaced by the donor bone marrow (reference 41). (E) 8 wk after reconstitution, the epidermal surface of the ears of the chimeric mice were either sensitized with DNFB (day 4 after DNFB) or left untouched (day 0). The number and origin (either host derived [CD45.1] or donor derived [CD45.2]) of the CD11c<sup>+</sup>ClassII<sup>+</sup> cells in the epidermal sheet isolated from the ears of the chimeric mice were determined by cell counting and FACS analysis. The mean  $\pm$  SD is shown ( $n = 3$  mice per group per time point). \*\*,  $P < 0.0001$  between the number of donor-derived WT CD11c<sup>+</sup>ClassII<sup>+</sup> cells and donor-derived *Gnaq*<sup>-/-</sup> CD11c<sup>+</sup>ClassII<sup>+</sup> cells in the epidermis of either group at either time point. There was no statistical difference in the number of host-derived WT resident LCs in the epidermis of either group at either time point. The data are representative of two independent experiments.

## 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  $\beta\gamma$  subunits, is required for chemotaxis (6). Likewise, experiments using the Gi inhibitor PTx (for review see reference 17) and our own experiments using  $G\alpha_{i2}$ -deficient cells (Table II) demonstrate that  $G\alpha_i$ -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

model (1) correctly postulates a central role for  $G\alpha_{i2}$  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 IP<sub>3</sub> generation and calcium release (35, 47–49), activation of NF- $\kappa$ B (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  $G\alpha_{i2}$ , although necessary, is not sufficient to induce chemotaxis of primary leukocytes to a large

**Table II.** Signaling requirements for chemotaxis

Receptor	Cell type (mouse)	G $\alpha_{12}$ dependent	G $\alpha_q$ 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	N	N	Classical
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.

array of chemoattractants. Instead, an additional, alternative G $\alpha_q$ -coupled pathway must be engaged before primary neutrophils and DCs can migrate (Table II), and this second, alternative G $\alpha_q$ -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 G $\alpha_q$ -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 G $\alpha_q$  is widely expressed in myeloid cells and DCs (60, 61) and that there is strong evidence that other Gq family members, such as G $\alpha_{15/16}$ , can mediate chemokine-induced IP<sub>3</sub> 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 G $\alpha_q$  is selectively targeted in a G $\alpha_{12}$ -sufficient cell and, to our knowledge, only a single study examining G $\alpha_q$ -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 G $\alpha_q$  is required for leukocyte chemotaxis to a subset of different chemokines, we also found that G $\alpha_q$  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 G $\alpha_q$ , and that activation of these PLC $\beta$  isoforms by G $\alpha_q$  leads to IP<sub>3</sub> generation and diacylglycerol production (64). However, it appears that the IP<sub>3</sub> response in fMLF- or IL-8-stimulated primary mouse bone marrow neutrophils is mediated largely, if not exclusively, by G $\alpha_{12}$ -containing G proteins, presumably through free  $\beta\gamma$ -mediated activation of one or more PLC $\beta$  isoforms. In fact, G $\alpha_q$  is not obligatorily required for the chemokine-induced early IP<sub>3</sub>-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, G $\alpha_q$  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 G $\alpha_q$ -activated calcium/cation

channel, it has been previously reported that calcium influx in fMLF-activated human neutrophils is linked, at least in part, to nonselective cation channels (46). We also know that calcium influx in response to chemokine receptor ligation is activated by CD38 and the calcium-mobilizing metabolites generated by CD38 (Table II). Finally, our data show that the calcium response to fMLF is indistinguishable in neutrophils that are deficient in  $G\alpha_q$  alone or that lack both  $G\alpha_q$  and cADPR, suggesting that  $G\alpha_q$  and CD38 are coregulators of the same cation/calcium channel.

We previously showed that extracellular 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  $G\alpha_q$  regulates neutrophil chemotaxis by calcium-independent mechanisms, our data strongly suggest that a major mechanism by which  $G\alpha_q$  regulates chemotaxis is by controlling the sustained calcium entry response. Our data also show that  $G\alpha_q$ -mediated calcium influx in response to fMLF stimulation is dependent on an initial intracellular calcium release controlled by  $G\alpha_{i2}$ . These results suggest that  $IP_3$ , generated by Gi-activated  $PLC\beta$ , sparks the global calcium response and, therefore, must be needed for calcium-dependent chemotaxis. However, published experiments showed that even when  $IP_3$  induction was completely abrogated in chemokine-stimulated neutrophils from  $PLC\beta2$  and  $PLC\beta3$  double-deficient mice, the chemotactic response of these  $PLC\beta$ -deficient cells remained intact (10). Thus, these published experiments suggest that neither  $IP_3$ -mediated intracellular calcium release nor calcium-dependent calcium influx are needed for neutrophil chemotaxis. Interestingly, the previously published experiments used “primed”  $PLC\beta$ -deficient neutrophils isolated from inflammatory sites (10), and we used bone marrow “naive” 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  $G\alpha_q$ -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  $G\alpha_q$ -coupled signaling pathway that regulates chemokine receptor signal transduction and chemotaxis. Most importantly, our data show that this novel  $G\alpha_q$ -dependent pathway plays a critical and nonredundant role in cell migration. However, although the  $G\alpha_q$ -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  $G\alpha_q$ -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<sup>+</sup> congenic B6, C57BL/6J.129 *Cd38*<sup>-/-</sup> (N12 backcross to C57BL/6J) (28), *Gnaq*<sup>-/-</sup> (N>5 backcross to C57BL/6J) (70), and *Gnai2*<sup>-/-</sup> (N>5 backcross to C57BL/6J) (70) mice were bred and maintained in the Trudeau Institute breeding facility. *Gnaq*<sup>-/-</sup> 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*<sup>-/-</sup>, or *Gnai2*<sup>-/-</sup> bone marrow 8 wk previously. This approach allowed us to examine the impact of  $G\alpha_q$  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  $^{137}\text{Cs}$  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, fMLF, PTx, and DNFB), Calbiochem (2-APB), and BD Biosystems (all antibodies). 8Br-cADPR (provided by T. Walseth, 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*<sup>-/-</sup> 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<sup>+</sup>), T cells (CD3<sup>+</sup> and CD4<sup>+</sup> or CD8<sup>+</sup>), myeloid DCs (CD11c<sup>+</sup>CD11b<sup>+</sup>CD8 $\alpha$ <sup>-</sup>), lymphoid DCs (CD11c<sup>+</sup>CD11b<sup>-</sup>CD8 $\alpha$ <sup>+</sup>), plasmacytoid DCs (CD11c<sup>int</sup>GR1<sup>+</sup>B220<sup>int</sup>), and LCs (CD11c<sup>+</sup>I-Ab<sup>+</sup>Langerin<sup>+</sup>) was determined.

To purify bone marrow neutrophils, bone marrow cells were first stained with biotinylated anti-GR1 antibody and MACS streptavidin microbeads (Miltenyi Biotec) and then positively selected on a MACS midi column. CD4 T cells were purified using CD4 microbeads (Miltenyi Biotec). 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 (Costar) with either a 3- $\mu\text{m}$  (for neutrophils) or 5- $\mu\text{m}$  (DCs and T cells) pore size polycarbonate filter. In some experiments, the isolated cells were first pretreated for 20 min with 100  $\mu\text{M}$  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- $\mu\text{m}$  fluorescent microbeads that were simultaneously counted on the flow cytometer. In some cases, the results are expressed as the mean  $\pm$  SD of the chemotactic index (CI). The CI represents the fold increase in the number of migrated cells in response to chemoattractants over the spontaneous cell migration (to control medium).

**Calcium mobilization assays.**  $10^7$  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 mean 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<sup>+</sup> B6 recipient mice were lethally irradiated and reconstituted with either CD45.2<sup>+</sup> B6 bone marrow or with CD45.2<sup>+</sup> *Gnaq*<sup>-/-</sup> 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 ClassII to determine whether the LCs (CD11c<sup>+</sup>ClassII<sup>+</sup>) were of donor (CD45.2<sup>+</sup>) or host (CD45.1<sup>+</sup>) 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<sup>+</sup>) and host-derived (CD45.1<sup>+</sup>) LCs (CD11c<sup>+</sup>ClassII<sup>+</sup>) present in the skin samples was then determined by cell counting and FACS.

To track DC migration from inflamed skin to LNs, 25  $\mu\text{l}$  FITC (8 mg/ml in 1:1 acetone/dibutylphthalate) was applied to two shaved abdominal areas of B6 or *Gnaq*<sup>-/-</sup> (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<sup>hi</sup>CD11c<sup>+</sup>classII<sup>+</sup> DCs was determined.

**Immunohistology.** 5- $\mu\text{m}$  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 $\times$  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  $\pm$  SD.

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