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Defective Macrophage Migration in Gαi2- but Not Gαi3-Deficient Mice

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Various heterotrimeric Gα proteins are considered to be involved in cell migration and effector function of immune cells. The underlying mechanisms, how they control the activation of myeloid effector cells, are not well understood. To elucidate isoform-redundant and -specific roles for Gα proteins in these processes, we analyzed mice genetically deficient in Gαi2 or Gαi3. First, we show an altered distribution of tissue macrophages and blood monocytes in the absence of Gαi2 but not Gαi3. Gαi2-deficient but not wild-type or Gαi3-deficient mice exhibited reduced recruitment of macrophages in experimental models of thioglycollate-induced peritonitis and LPS-triggered lung injury. In contrast, genetic ablation of Gαi3 had no effect on Gαi-dependent peritoneal cytokine production in vitro and the phagocytosis-promoting function of the Gαi-coupled C5a anaphylatoxin receptor by liver macrophages in vivo. Interestingly, actin rearrangement and CCL2- and C5a anaphylatoxin receptor-induced chemotaxis but not macrophage CCR2 and C5a anaphylatoxin receptor expression were reduced in the specific absence of Gαi2. Furthermore, knockdown of Gαi2 caused decreased cell migration and motility of RAW 264.7 cells, which was rescued by transfection of Gαi2 but not Gαi3. These results indicate that Gαi2, albeit redundant to Gαi3 in some macrophage activation processes, clearly exhibits a Gαi3 isoform-specific role in the regulation of macrophage migration.


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Only the rescue of Gα12 but not Gα13 restored chemotaxis of RAW 264.7 cells that were silenced for Gα12, thus providing, to our knowledge, the first definitive evidence for an isoform-specific role of Gα12 in the regulation of macrophage migration.

Materials and Methods

Mice

Generation of Gα12- and Gα13-deficient SV129 mice was described previously (13, 24). These Gα knockout (KO) mouse strains were back-crossed to C57BL/6J (B6) mice for >11 generations and were strictly kept under UV conditions. Under these conditions, no signs of intestinal inflammation were visible in Gα12-deficient mice during the course of the study (15). C5αR-deficient mice backcrossed to C57BL/6J mice for six generations were provided by C. Gerard (Boston, MA) (25). B6 control mice were from Charles River Laboratories. All mice were used at 8–14 wk of age. Animal experiments were conducted in accordance with current laws in combination with the regulations of the local authorities.

Monocyte/macrophage cell counts and flow cytometry

CD11b-positive liver macrophages as well as lavaged macrophages from lung and peritoneum were isolated and quantitated as described previously (26–28). Blood was obtained from anesthetized mice by puncture of the retro-orbital plexus, transferred to EDTA-coated tubes, and counted for blood monocytes by the automated Animal Blood Counter (Scil Animal Care, Vierenheim, Germany). Absolute cell numbers of CD11b+Ly6C−/CD62L− monocyte subsets were obtained by using BD Truecount counting beads (BD Pharmingen, San Diego, CA). Expression of CD11b, L-selectin (CD62L) adhesion molecules on F4/80+positive blood cells was analyzed with allophycocyanin-conjugated anti-CD11b/CD62L mAbs by flow cytometry, using a FACScalibur flow cytometer (BD Biosciences, Heidelberg, Germany).

Thiglycollate-induced peritonitis

Peritoneal recruitment of leukocytes was induced using 4% thioglycollate (Life Technologies). After 4 d, mice were killed, and the peritoneal cavity was rinsed 10 times with 1 ml ice-cold PBS/5 mM EDTA. Total cell count of the collected peritoneal lavage fluid was assessed. For quantification of macrophage influx, differential cell counts were performed on cytospins (10 min at 55 °C) stained with May–Gruenwald–Giemsa, using 300 μl peritoneal lavage fluid.

LPS-induced lung inflammation

Mice were anesthetized with ketamine and xylazine, the trachea was cannulated, and 20 μg Escherichia coli LPS (Sigma-Aldrich) was applied. Control animals received PBS instead of LPS. Mice were killed at 4 d, and bronchoalveolar lavage was performed five times with 1 ml PBS containing 5 mM EDTA at 4°C. For quantification of alveolar macrophage accumulation, differential cell counts were performed on cytospins (10 min at 55 °C) stained with May–Gruenwald–Giemsa using 300 μl bronchoalveolar lavage fluid.

In vivo erythropagocytosis by liver macrophages

CsA-regulated phagocytosis was induced by a single i.p. injection of the pathogenic anti-erythrocyte 34–3C mAb (IgG2a, 150 μg/mouse), as described previously (26, 28, 29). Mice were sacrificed 2 d later, and the livers were processed for histological examination. Tissues were fixed in 10% buffered formalin, embedded in paraffin, and stained with H&E, according to conventional procedures.

LPS-activated induction of Gα12-deficient macrophages in vitro

Peritoneal macrophage (PM) cells were flushed out of the peritoneal cavity of Gα12-deficient or wild-type (WT) B6 mice, washed twice with PBS/5 mM EDTA, and suspended in RPMI 1640 medium/10% FCS. The PM cells were allowed to adhere for 4 h in 12-well plates (Corning) at 37°C, washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml). For visualization of cell morphology, PM cells were fixed for 2 h with PFA (0.5% (vol/vol)) and then incubated with fibronectin and incubated at 37°C overnight. Next day, cells were washed and then stimulated for 4 h with C5a (50 ng/ml)
whether the presence of Gαi2 and Gαi3 expression vectors using Lipofectamine 2000 (Invitrogen, St. Louis, MO) as delivery cargo. After 2 d, the transfectants were used in functional Transwell migration and immunoblot assays as described before.

Statistical analysis

To analyze differences between more than two normally distributed groups, a one-way ANOVA was performed. Pairwise comparisons were then performed using Tukey’s test. To analyze differences between two normally distributed groups, an unpaired Student t test was used.

Results

Defective homeostatic and inflammation-induced migration of macrophages in Gαi2-deficient mice

Heterotrimeric G proteins of the Gα family have been implicated in signaling pathways regulating immune homeostasis and cell migration. Previously, it was shown that lack of Gαi2 leads to defects in the trafficking of T and B cells to lymph nodes (18, 19) and in the recruitment of cosinophils and neutrophils to sites of inflammation (20, 22, 23). Moreover, increased blood monocyte numbers were noted in Gαi2-deficient mice, whereas monocytes in the bone marrow (BM) are not affected (Ref. 22; data not shown). To determine the potential role of the individual Gα proteins in the homing of monocytes and/or macrophages, we first examined whether the presence of Gαi2 and Gαi3 is needed for proper distribution of macrophages at different organ sites. Macrophage cell counts in lung, peritoneum, and liver were similar between WT B6 mice and Gαi2-deficient mice (Gαi3 KO). In contrast, Gαi2 KO mice exhibited significantly reduced numbers of tissue macrophages in the compartments tested (Fig. 1A) and increased numbers of total and CD115+ monocytes in peripheral blood (Fig. 1B, left and middle panels, respectively). The expression pattern of CCR2 and the adhesion molecules CD11b and CD62L on monocytes, however, appeared normal in Gαi2 KO, Gαi3 KO, and WT B6 mice (Fig. 1C).

At least two distinct peripheral blood monocyte subpopulations are recognized in mice (34), and the Ly6C++ inflammatory monocyte subset specifically expresses CCR2 and CD62L (35). Increased accumulation of this Ly6C++ inflammatory monocyte subset was also evident in Gαi2 KO mice, suggesting that Gαi2—but not Gαi3—is required for homeostatic and inflammation-induced migration of monocytes and macrophages.

Therefore, we next examined the relative contribution of Gαi2 and Gαi3 in mediating the recruitment of macrophages in two models of acute inflammation, namely thioglycollate-induced peritonitis and LPS-induced alveolitis. Macrophage accumulation to the peritoneum and lung was studied at day 4 by counting macrophage numbers obtained after peritoneal and bronchoalveolar lavages. As shown in Fig. 2A, Gαi2 KO mice showed significantly decreased macrophage elicitation in response to both stimuli, whereas Gαi3 KO mice displayed either normal (thioglycollate) or increased (LPS) macrophage numbers.

We also tested the migratory capacity of Gαi2-deficient macrophages in response to CCL2 and C5a in standard Transwell chemotaxis assays in vitro (Fig. 2B). Similar to their defect to migrate into inflamed tissue in vivo, PM from Gαi2 KO mice showed reduced CCL2/C5a-induced chemotaxis by CCR2 and C5aR in vitro, as compared with Gαi3 KO and WT PM cells (Fig. 2B, left panel). Recent analyses of lymphocyte migration suggested Gαi-dependent changes in the expression of CC and CXC chemokine receptors regulating the movement of CD4 and CD8 T cells (18). Examining Gαi-deficient PM cells, we analyzed in

**FIGURE 1.** Altered distribution of tissue macrophages and blood monocytes in the genetic absence of Gαi2 but not Gαi3. (A) Macrophages were obtained from the indicated organ sites of B6 and Gαi2-, or Gαi3 KO mice and counted as described in Materials and Methods. (B) Blood was collected and analyzed for circulating monocytes by an automatic ABC blood counter (left panel) or by counting beads in respect to monocyte markers, CD115 and Ly6C (middle and right panels, respectively). (A and B) The mean cell count ± SEM (n = 15 mice for each group) is shown. (C) Peripheral blood monocytes from B6 (upper panels), Gαi2 KO (middle panels), and Gαi3 KO mice (lower panels) were analyzed for the surface expression of CD11b, CD62L, and CCR2 by flow cytometry. One representative experiment out of three is shown. *p < 0.05, **p < 0.001, ***p < 0.0005.
this study the expression status of C5aR and CCR2 on macrophages by flow cytometry. We found no altered cell surface appearance of the two receptors on these cells from Ga\textsubscript{i2} KO and Ga\textsubscript{i3} KO mice, as compared with WT controls (Fig. 2B, right panel). Irrespective of normal C5aR cell surface expression, fluorescence microscopic analysis of Ga\textsubscript{i}-deficient PM cells revealed an impaired C5a-induced cell polarization response in the selective absence of Ga\textsubscript{i2} but not Ga\textsubscript{i3} (Fig. 2C). Because C5aR is known to couple to both Ga\textsubscript{i2} and Ga\textsubscript{i3}, this result indicates a specific role of Ga\textsubscript{i2} in C5aR signaling that leads to actin rearrangement and cell migration, which cannot be compensated by Ga\textsubscript{i3}.

Normal LPS-induced cytokine release and C5aR-regulated erythrophagocytosis in Ga\textsubscript{i2}-deficient mice

Ga\textsubscript{i}-dependent signaling has been shown to play a crucial role in LPS-induced cell activation (36). Thus, we next examined the capacity of Ga\textsubscript{i2} and Ga\textsubscript{i3}-deficient macrophages to respond to LPS by measuring the release of inflammatory mediators. PM cells were cultured overnight, incubated with LPS (1 \(\mu\)g/ml) for 12 h, and analyzed for the production of TNF-\(\alpha\) and CXCL2 by ELISA. High levels of these cytokines were detectable in Ga\textsubscript{i2} KO PM cells upon LPS stimulation (Fig. 3A). Ga\textsubscript{i3} KO PM cells were equally effective, whereas inactivation of both Ga\textsubscript{i} isoforms by PTx caused an impaired response (Fig. 3A). Taken together with the observation that macrophages express Ga\textsubscript{i2} in a stoichiometric excess over Ga\textsubscript{i3} (23), the results indicate quantitative differences but functional redundancy of the two Ga\textsubscript{i} isoforms in LPS-induced macrophage activation.

To further assess the specific role of Ga\textsubscript{i2} or Ga\textsubscript{i3} in macrophage-mediated immune reactions in vivo, we used the IgG-mediated model of hemolytic anemia. WT, Ga\textsubscript{i2} KO, or Ga\textsubscript{i3} KO mice were injected with 150 \(\mu\)g anti-erythrocyte band 3 (34-3C) self-reactive Abs, and ingestion of IgG-bound RBC by liver macrophages was monitored as described previously (29, 37, 38). In this type of autoimmune disease model, cellular RBC destruction is induced by simultaneous activation of IgG FcRs and the Ga\textsubscript{i}-coupled C5aR on Kupffer cells (26, 28). In agreement with the previous observations, WT B6 mice showed a detectable phagocytic response upon i.p. injection of the 34-3C autoantibody at day 2, whereas KO mice lacking C5aR (C5aR KO) showed reduced RBC destruction (Fig. 3B). In contrast to C5aR KO mice, liver phagocytosis was not significantly ameliorated in Ga\textsubscript{i2}- or Ga\textsubscript{i3}-deficient animals (Fig. 3B). These results suggest that C5a-induced chemotaxis in vitro (see Fig. 2B) but not C5aR-
regulated phagocytosis of IgG-bound RBCs in vivo is mediated by a Gαi2-selective mechanism.

KD of Gαi2 in macrophages causes defective chemotaxis that is rescued upon Gαi2 but not Gαi3 transfection

Among various Gα-dependent macrophage functions tested, we identified chemotactic migration to be selectively regulated by Gαi2. To further strengthen our findings on the significance of Gαi2 specificity for cellular migration, we generated a stable KD of Gαi2 in RAW 264.7 macrophages by Gαi2 shRNA expression. These Gαi2 KD macrophages showed markedly reduced Gαi2 mRNA levels (Fig. 4A, left panel) and a reduction of protein expression by ∼75% (Fig. 4B, left panel). Interestingly, we neither observed a concomitant upregulation of Gαi3-specific transcripts (Fig. 4A, right panel) nor of Gαi3 protein (Fig. 4B, right panel). Corresponding to decreased expression of Gαi2, the expression levels of the predominant GB isoforms, GB1, and GB2, were also decreased in Gαi2 KD macrophages (Fig. 4C).

Having established selective inhibition of Gαi2 protein expression, we then analyzed the Gαi2 KD effect on cytokine production and cell migration. Comparable to the situation with primary PM cells (Fig. 3A) and mock-transfected cells (Fig. 5A), LPS-induced production of TNF-α and CXCL2 was still detectable in Gαi2 KD macrophages (Fig. 5A). Importantly, however, both chemokinesis (Fig. 5B, white bars) and C5a-induced chemotaxis (Fig. 5B, black bars) were markedly reduced in Gαi2 KD cells as compared with mock-transfected cells confirming a selectivity of Gαi2 for this cellular response. To rigorously check our finding on Gαi2-selective inhibition of Gαi2 protein expression, we then analyzed the Gαi2 KD effect on cytokine production and cell migration.
tive regulation of chemotactic migration, we finally performed rescue experiments in KD cells transfrcted with Gα2 or Gα3 cDNAs. Importantly, Gα2 KD macrophages regained the ability for C5a-induced chemotaxis only in the presence of cDNA expression vectors encoding for Gα2 (pGα2) but not Gα3 (pGα3) (Fig. 5C).

Discussion

We have demonstrated previously that, despite the high homology between Gα2 and Gα3, the inflammatory cascade in the lung Arthus reaction critically depends on Gα2 but not Gα3 (23). The present work significantly extends these findings by providing novel evidence that Gα2 plays a critical role in the homeostatic distribution of tissue macrophages (Fig. 1) as well as inflammation-induced migration in vivo (Fig. 2A). The in vitro observation that Gα2 but not Gα3 is essential for optimal CCL2- and C5a-induced migration (Fig. 2B) further indicates that the reduced recruitment of macrophages in acute inflammation in Gα2-deficient mice may be due to a Gα2-specific defect in directed chemotaxis of these cells. However, not all effector functions of macrophages are mediated by a Gα2-specific mechanism, and Gα3 is able to substitute for Gα2 in C5aR-regulated phagocytosis as well as Gα2-dependent cytokine production (Fig. 3). The latter observation is in agreement with the findings of Fan et al. (39), who suggested that Gα2 and Gα3 are both involved in LPS-induced cell activation. However, we also note that Gα3-deficient mice exhibit an increased accumulation of macrophages in lung tissue in response to LPS (Fig. 2A), indicating that Gα3 may negatively influence certain Gα2 signaling events. Further studies will be needed to test the possibility of an antagonistic interplay between Gα2 and Gα3 in tissue infiltration of macrophages, as has been suggested before for CXCR3-mediated signaling in T cells (40).

Macrophages are present in all tissues, and many of these cells are replaced continually from BM-derived monocytes. A recent study suggested that tissue macrophages can also develop from a nonhematopoietic origin in a Myb-independent manner (41). We do not discriminate between the different origins of macrophages, but we found a reduction of total macrophage counts in lung, liver, and peritoneum in the absence of Gα2 (Fig. 1A). Interestingly, Gα2-deficient mice show increased blood monocyte numbers (Fig. 1B), thus indicating an involvement of Gα2 in the transit of monocytes from blood into tissues. Previous studies suggested a role of CCL2 in the homeostatic migration of the CCR2-positive Ly6C hi monocyte subpopulation (42, 43). CCR2-deficient mice showed reduced Ly6C hi monocyte numbers in the blood but not in the BM, indicating an involvement of CCR2 in steady-state BM egress (44). Gα2 deficiency, while causing reduced CCR2-mediated macrophage chemotaxis (Fig. 2B), is associated with increased numbers of peripheral blood Ly6C hi monocytes (Fig. 1B), indicating that homeostatic emigration of this inflammatory monocyte subset from the BM can occur in the absence of Gα2-mediated CCR2 activation.

Changes in the expression of GPCRs are thought to be critical for the migration of immune cells. Previously, a Gαi protein requirement in the regulation of CC and CXCR expression was reported for T lymphocytes (18). Gα2-deficient CD4 and CD8 T cells each displayed reduced levels of CCR7 and CXCR4 as well as CD62L. Importantly, our results demonstrate comparable expression profiles of CD11b, CD62L, and CCR2 on blood monocytes (Fig. 1C) and of CCR2 and C5aR on peritoneal macrophages (Fig. 2B) in WT, Gα2, and Gα3 KO mice. Although Gα2-deficient macrophages normally express CCR2 and C5aR on their cell surfaces, they display a severe defect of CCL2- and C5a-induced chemotaxis (Fig. 2B). Actin reorganization and cell polarization are required events for directional cell movement (45, 46). To our knowledge, our data now show for the first time that both C5a-induced remodeling of the actin cytoskeleton and chemotaxis are strictly dependent on the presence of Gα2 in macrophages (Fig. 2C).

The diminished chemotactic response exhibited by mice lacking Gα2 but not Gα3 may depend on differences in the total amount of the two Gαi proteins. Macrophages, like most other cell types (16), express Gα2 in much higher concentrations than Gα3 (23). Importantly, however, low Gα3 can compensate for the lack of high Gα2 in LPS-induced macrophage activation and C5αR-regulated erythrophagocytosis (Fig. 3), suggesting that structural rather than quantitative differences of Gα2 and Gα3 determine the unique function of Gα2 in the regulation of macrophage mi-
gration. Our RNA interference data support this conclusion. KD of Goi2 in RAW 264.7 macrophages, while not affecting Gs3 expression (Fig. 4) and LPS-induced production of TNF-α and CXCL2 (Fig. 5A), causes decreased Gβ1 and Gβ2 protein levels (Fig. 4) and defective chemotaxis (Fig. 5B). Most significantly, only the overexpression rescues of Goi2 but not Gs3 restore migration of Goi2-silenced RAW 264.7 cells (Fig. 5C).

In summary, we have shown that Goi2 is a crucial regulator of macrophage migration and the specific KD of Goi2 reflects the phenotype of Goi2-deficient PM cells with respect to chemotaxis. Importantly, the rescue of Goi2 but not Gs3 could restore this phenotype providing clear evidence for an isoform-specific role of Goi2. In contrast, Goi2 was able to substitute for Goi2 regarding the LPS-induced cytokine production and the phagocytic activity of liver macrophages. These findings serve as an important basis for the identification of the underlying mechanisms of redundancy versus isoform specificity of Goi2 and Goi3. Such mechanisms could be based on a different subcellular localization of these isoforms (16, 47), structural differences in regions different from the receptor- or effector-binding domains, as well as the total amount of Goi proteins in the cells, with the latter obviously not playing a decisive role in our model.

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Disclosures
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