Knockout mouse models reveal the contributions of G protein subunits to complement C5a receptor–mediated chemotaxis

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Running title: G proteins and chemotactic signaling
G protein–coupled receptor (GPCR) signaling is required for the navigation of immune cells along chemoattractant gradients. However, chemoattractant receptors may couple to more than one type of heterotrimeric G protein, each of which consists of a Gα, Gβ, and Gγ subunit, making it difficult to delineate the critical signaling pathways. Here we used knockout mouse models and time-lapse microscopy to elucidate Gα and Gβ subunits contributing to complement C5a receptor–mediated chemotaxis. Complement C5a–mediated chemokinesis and chemotaxis were almost completely abolished in macrophages lacking Gnai2 (encoding Gαi2), consistent with a reduced leukocyte recruitment previously observed in Gnai2–/– mice, whereas cells lacking Gna13 (Gα13) exhibited only a slight decrease in cell velocity. Surprisingly, C5a-induced Ca2+ transients and lamellipodial membrane spreading were persistent in Gnai2–/– macrophages. Macrophages lacking both Gnaq (Gα4) and Gna11 (Gα11), or both Gna12 (Gα12) and Gna13 (Gα13) had essentially normal chemotaxis, Ca2+ signaling, and cell spreading, except Gna12/Gna13-deficient macrophages had increased cell velocity and elongated trailing ends. Moreover, Gnaq/Gna11-deficient cells did not respond to purinergic receptor P2Y2 stimulation. Genetic deletion of Gna15 (Gα15) virtually abolished C5a-induced Ca2+ transients, but chemotaxis and cell spreading were preserved. Homozygous Gnb1 (Gβ1) deletion was lethal, but mice lacking Gnb2 (Gβ2) were viable. Gnb2–/– macrophages exhibited robust Ca2+ transients and cell spreading, albeit decreased cell velocity and impaired chemotaxis. In summary, complement C5a–mediated chemotaxis requires Gα2 and Gβ2, but not Ca2+ signaling, and membrane protrusive activity is promoted by G proteins that deplete phosphatidylinositol 4,5-bisphosphate.

Introduction

The study of immune cell chemotaxis dates back to 1888 (1), when the German ophthalmologist Th. Leber (Theodor Karl Gustav Leber), using a model of mycotic keratitis, observed the migration of leukocytes to sites of inflammation, where they accumulated in large numbers, reminiscent of the swarming of neutrophils to injured tissue described more recently by Lämmermann et al. (2). Gradients of chemoattractant ligands emanating from sites of inflammation or injured tissue guide cells by binding to G protein-coupled receptors (GPCRs), but how spatiotemporal ligand-GPCR signaling accomplishes this has not been delineated. Dictyostelium discoideum has proven to be a good model system for the study of chemotaxis (3), leading to the development of the local-excitation, global-inhibition (LEGI) model (4) and more complex excitatory network hypotheses (5). In contrast to Dictyostelium amoebae, mammalian chemoattractant receptors, such as the complement C5a receptor (C5aR), may activate more than one type of heterotrimeric G protein (6-8), which adds considerable complexity to the signal transduction. Nevertheless, the Gαi/o family has been strongly implicated in chemotactic signaling since pertussis toxin (PTX; previously known as lymphocytes promoting factor) from Bordetella pertussis, which blocks the activation of Gαi/o subunits by GPCRs, inhibits the chemotaxis of macrophages and other phagocytes (9-11). Moreover, the recruitment of neutrophils and macrophages to inflamed lung or peritoneum is decreased by around 50% in mice lacking Gnai2, which encodes Gα2 (12,13).

Genetic deletion of Gna15, a hematopoietic-specific gene coding for the Gαq/Gα11 family member Gα15, markedly decreases complement C5a-induced Ca2+ transients in macrophages (14), but the role of Gα15 in complement C5a-mediated chemotaxis has not been determined. The other members of the Gαq/Gα11 family are encoded by Gnaq (Gαq) and Gna11 (Gα11), respectively, in mouse, but the roles of these subunits in chemotaxis are unclear. In cotransfection studies, the chemokine (chemotactic cytokine) receptors for CCL2 (chemokine (C-C motif) ligand 2; also known as monocyte chemoattractant protein-1) and CCL5 (also known as RANTES (regulated on activation, normal T cell expressed and secreted)) were shown to couple to Gαq (6). In contrast, CXCL8 (chemokine (C-X-C motif) ligand 8; also known as interleukin-8) and complement C5a did not activate Gαq (6). However, complement C5a may indirectly couple to Gαq and/or Gα11 through C5aR-induced autocrine ATP signaling which activates purinergic receptors (15,16).

In this study, we used knockout mouse models to explore the roles of different G protein subunits in macrophage chemotaxis. More specifically, we used time-lapse microscopy to visualize the navigation and movement of macrophages, isolated from wild-type (WT) or various knockout mice, in a chemotactic complement C5a gradient, and, in parallel, we imaged the Ca2+...
signaling and lamellipodial membrane dynamics in individual cells upon C5aR stimulation.

**Results**

**Gna12, but not Gna13, is critical for complement C5a-mediated chemotaxis**

RNA sequence analysis of purified resident peritoneal F4/80+ cells (macrophages) revealed that Gna12 (Gαi2) and Gna13 (Gαi3) of the Gαi/o family of Gα-subunits, as well as Gnas (Gαs), and members of the Gαd/Gα11 (Gnaq, Gna11, and Gna15) and Gα12/Gα13 (Gna12 and Gna13) families, are expressed, as well as complement C5a receptor 1 (as known as CD88), encoded by C5ar1 (Fig. 1A and B). Notably, the controversial C5aR, complement C5a receptor 2 (17), encoded by C5ar2, was negligibly expressed (see the inset with interrupted y-axis in Fig. 1A). We used knockout mouse models to explore the roles of the various Gα and Gβ subunits shown in Fig. 1B, except Gnas (Gαs), in transducing gradients of the chemoattractant complement C5a into stimulated motility (chemokinesis) and directed cell migration (chemotaxis) using the µ-Slide Chemotaxis chamber (16,18) and time-lapse, phase-contrast microscopy. Macrophages isolated from WT mice migrated robustly along chemotactic gradients of complement C5a (Fig. 1C; supplemental Videos S1 and S2). Incubation with PTX (1 µg/ml), which ADP ribosylates a serine residue and inhibits Gαi2 and Gαi3, as well as other Gαi/o family members (19), abolished complement C5a-mediated chemokinesis and chemotaxis (Fig. 1C). Similarly, genetic deletion of Gna12 markedly impaired cell velocity and chemotactic navigation in a complement C5a gradient (Fig. 1, C and D; supplemental Videos S3-S6), whereas deletion of Gna13 only marginally impaired cell velocity. The extent of in vitro chemotaxis impairment in Gna12−/− macrophages was greater than expected when compared to WT versus Gna12−/− mouse in vivo inflammation models (12,13).

**Complement C5a-induced Ca2+ transients and lamellipodial cell spreading are intact in Gna12-deficient macrophages**

We have previously shown that complement C5a induces robust Ca2+ transients (20), which are monophasic at high agonist concentrations, and lamellipodial cell spreading in mouse macrophages (21). In the following experiments, we simultaneously imaged intracellular [Ca2+] using the fluorescent Ca2+ indicator Cal-520, and membrane dynamics, assessed by brightfield microscopy (Fig. 2). Macrophages, like neutrophils, migrate on a two-dimensional surface in an amoeboid fashion in which the dominant lamellipodial membrane protrusion steers the cell (22,23), and in the case of chemotaxis needs to be directed towards higher concentrations of chemoattractant (24), as evident, for example, in supplemental Video S1. We therefore tested whether complement C5a-induced lamellipodial membrane protrusive activity is defect in Gna12−/− macrophages. Surprisingly, similar to WT macrophages, complement C5a induced robust monophasic Ca2+ transients and lamellipodial membrane protrusions in the absence of Gna12 (Fig. 2A; supplemental Videos S7 and S8). Deletion of either Gna12 or Gna13 did not significantly decrease the peak levels of complement C5a-induced Ca2+ transients or cell spreading, indexed as the peak projected cell area normalized to that preceding stimulation (Fig. 2B). The Ca2+ transient peak, but not peak cell spreading, was decreased by PTX treatment (Fig. 2B). The temporal relation between complement C5a-induced Ca2+ transients and cell spreading suggested that rapid increases in intracellular [Ca2+] may promote branched actin polymerization, which drives lamellipodial membrane extension. To test this notion, we loaded the cytosol of macrophages with the Ca2+ chelator EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) using its cell-permeable acetoxymethyl (AM) ester form (EGTA/AM). Intracellular loading of cells with EGTA almost completely abolished complement Ca2+-induced Ca2+ transients (Fig. 3A), but cell spreading was not abrogated (Fig. 3B). This implies that the second messenger Ca2+ is not important for the formation of complement C5a-induced lamellipodial membrane protrusions.

**Double Gnaq/Gna11 or Gna12/Gna13 knockout macrophages have robust complement C5a-mediated chemotaxis**

Next, we investigated the roles of the Gαd/Gα11 and Gα12/Gα13 families of Gα subunits (schematically illustrated in Fig. 4A) in complement C5a-mediated chemotaxis using triple mutant mice in which one gene is deleted and second one is conditionally knocked out in myeloid cells, which includes macrophages. The median velocity and chemotaxis index of macrophages isolated from Gnaq−/−/LysoM-Cre/Gna11−/− (Gnaq/Gna11 dKO (double knockout)) mice, in which Gna11 is constitutively deleted and Gnaq is conditionally deleted, did not differ from the values for WT
macrophages (Fig. 4B). Similarly, macrophages isolated from Gna12−/−/Gna13−/−/LysM-Cre (Gna12/Gna13 dKO) mice, in which Gna12 is deleted and Gna13 is conditionally deleted, clearly navigated well along chemotactic complement C5a gradients, although the median velocity of Gna12/Gna13 dKO macrophages was modestly increased (Fig. 4, B and C). The polarized morphology of Gnaq/Gna11 dKO macrophages migrating in a complement C5a gradient was unremarkable (Supplemental Videos S9 and S10), whereas Gna12/Gna13 dKO macrophages exhibited modestly elongated trailing ends (Fig. 4D; supplemental Videos S11 and S12), reminiscent of the phenotype of conditional Rhoc knockout macrophages (25), but much less extreme than Rhoc/Rhob double knockout macrophages (25). Measurements of maximal tail length (Fig. 4, D and E), assessed over a 6-h migration period, confirmed that Gna12/Gna13 dKO macrophages developed elongated trailing ends (Fig. 4E). The modest phenotypes of Gna12/Gna13 dKO macrophages suggest that complement C5a coupling to Ga12 and Ga13 contributes to the activation of the Rho subfamily of Rho GTPases and retraction of the trailing end in migrating cells. Consistent with this interpretation, we found that application of complement C5a increased the levels of active RhoA (RhoA-GTP) in wild-type mouse bone marrow-derived macrophages, measured by G-LISA assays (Fig. 4F).

**UTP-induced Ca2+ transients are largely abolished in Gnaq/Gna11 double knockout macrophages**

Extracellular uridine 5′-triphosphate (UTP) and adenosine 5′-triphosphate (ATP) induce large Ca2+ transients in mouse macrophages by binding to P2Y2 receptors (P2Y2Rs), although ATP additionally induces Ca2+ influx via P2X receptors, ATP-gated, non-selective cation channels (20). P2Y2Rs are thought to signal to G proteins which are typically not distinguished by receptors (8). Indeed, we found that UTP-induced Ca2+ signaling was almost completely abrogated in Gnaq/Gna11 dKO macrophages, whereas subsequent application of complement C5a induced robust Ca2+ transients (Fig. 5, A and B). Note that the decaying Ca2+ hump following large complement C5a-induced Ca2+ peaks can be explained by store-operated Ca2+ entry since it is absent in Ca2+-free media and can be evoked by reintroduction of Ca2+-containing media (20). The large complement C5a-induced Ca2+ peak persists in Ca2+-free media and is produced by endoplasmic reticular (ER) Ca2+ release (20).

Unlike Gaq and Ga11, which stimulate phospholipase C-β (PLC-β) activity and generate the endoplasmic reticular Ca2+ releasing agonist inositol 1,4,5-trisphosphate (IP3) via phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis, Ga12 and Ga13 do not stimulate PLC-β, but instead activate Rho guanine nucleotide exchange factors (RhoGEFs). Thus, complement C5a signaling to Ga12 and Ga13 is unlikely to shape the Ca2+ response. Indeed, complement C5a induced robust Ca2+ transients in Gna12/Gna13 dKO macrophages (Fig. 6, A and B). There were no significant differences in the peak Ca2+ responses of Gna12/Gna13 dKO or Gnaq/Gna11 dKO macrophages compared to wild-type cells (Fig. 6C). Moreover, complement C5a induced lamellipodial membrane spreading in both Gnaq/Gna11 dKO and Gna12/Gna13 dKO macrophages, such that median cell area significantly increased by 1.5-fold (n = 25; 3 independent experiments) and 1.4-fold (n = 17; 2 independent experiments), respectively.

**Gna15−/− macrophages have intact chemotaxis, but complement C5a-induced Ca2+ signaling is largely abolished**

To determine whether the hematopoietic-restricted α-subunit Ga15, a member of the Gaq/Ga11 family (as illustrated in Fig. 7A), is required for macrophage migration towards complement C5a we performed real-time chemotaxis assays. Similar to WT cells, Gna15−/− macrophages migrated efficiently along complement C5a gradients (Fig. 7B; supplemental Videos S13 and S14) and cells showed normal polarized morphologies in a chemotactic gradient (Fig. 7C; supplemental Videos S13 and S14). There were no significant differences in the measured velocity and chemotactic efficiency of WT and Gna15−/− macrophages migrating in a complement C5a gradient (Fig. 7D).

Davignon et al. (14) showed that Gna15 is not required for normal hematopoiesis, but found that complement C5a-induced Ca2+ transients, averaged from at least 5 cells, were markedly decreased in thioglycolate-elicted peritoneal macrophages from Gna15−/− mice, whereas responses to ATP or UTP were similar to WT macrophages. In accord with the findings of Davignon et al. (14), we found that WT resident peritoneal macrophages produced robust Ca2+ transients upon application of complement C5a, as well as 10 min later following the application of UTP (Fig. 8A), whereas most individual
Gna15\textsuperscript{-/-} macrophages did not respond to complement C5a and those which responded gave a weak signal (Fig. 8, B and C). The peak intracellular Ca\textsuperscript{2+} response to UTP 10 min after complement C5a application was significantly weaker in WT macrophages compared to Gna15\textsuperscript{-/-} macrophages (Fig. 8C). This could possibly be explained as follows: The large cytosolic Ca\textsuperscript{2+} signal induced by complement C5a in WT cells (which is virtually absent in Gna15\textsuperscript{-/-} cells) causes inactivation of ER Ca\textsuperscript{2+} release channels (IP\textsubscript{3} receptors), which has been shown to occur after ~30 s in the sustained presence of IP\textsubscript{3} (28). Thus, the weaker response to the second agonist (UTP) probably reflects insufficient resensitization of these channels and/or other factors, such as insufficient time for replenishment of ER Ca\textsuperscript{2+} stores and membrane PIP\textsubscript{2} levels.

**Complement C5a-induced lamellipodial membrane protrusions are preserved in Gna15\textsuperscript{-/-} macrophages**

We next tested whether impaired lamellipodial cell spreading accompanied the loss of Ca\textsuperscript{2+} signaling in Gna15\textsuperscript{-/-} macrophages. Instead of using transmitted light on the spinning disk confocal microscope to assess membrane dynamics, as in earlier experiments, we used the fluorescent plasma membrane stain CellMask Orange. This fluorophore nicely stained the membrane of macrophages, but it also weakly labeled the surface of fibronectin-coated µ-Slide I chambers (Fig. 9A). Application of complement C5a induced rapid and robust lamellipodial membrane protrusions in WT (Fig. 9A; supplemental Video S15) and Gna15\textsuperscript{-/-} macrophages (Fig. 9A; supplemental Video S16). Fig. 9A shows snapshots of CellMask Orange recordings, whereas the supplemental videos (supplemental Videos S15 and S16) show an overlay of fluorescent Cal-520 and CellMask Orange signals. A sharp increase in intracellular [Ca\textsuperscript{2+}] precedes cell spreading in WT macrophages (supplemental Video S15), whereas no increase in intracellular [Ca\textsuperscript{2+}] is seen in Gna15\textsuperscript{-/-} macrophages (see the Cal-520 fluorescence intensity trace in Fig. 9A and supplemental Video S16). Notably, in Fig. 9A and in the accompanying video (supplemental Video S15), the introduction of complement C5a-containing medium to WT macrophages caused a marginal shift of the focal plane in the z-axis. Quantification of the images confirmed that complement C5a induced significant increases in projected cell area in both WT and Gna15\textsuperscript{-/-} macrophages (Fig. 9B), and the effects could not be explained by effects of shear stress (Fig. 9C), which typically causes ruffling with minimal net cell spreading. There was no difference, WT versus Gna15\textsuperscript{-/-} macrophages, in the magnitude of complement C5a-induced cell spreading (Fig. 9D).

**Gnb1 and Gnb2 knockout mouse models generated using CRISPR/Cas9**

Impaired inflammation-mediated phagocyte recruitment in Gna12\textsuperscript{-/-} mice (12, 13) and real-time chemotaxis assays using macrophages isolated from Gna12\textsuperscript{-/-} mice (see Fig. 1) strongly isolate Ga\textsubscript{a} as a central signal transducer in chemotactic complement C5a signaling. In addition to the inhibitory effect of Ga\textsubscript{a} subunits on adenylcyclase, the G\βγ subunits released upon activation of members of the Ga\textsubscript{a} family are known to regulate various signal pathways, including PLC-β isoforms, phosphoinositide 3-kinases (PI3Ks), and ion channels (8). PI3Ks catalyze the conversion of PIP\textsubscript{2} to phosphatidylinositol 3,4,5-trisphosphate (PIP\textsubscript{3}), a key phospholipid implicated in chemotactic signaling (3). G\β subunits are encoded by five genes in mouse (Gnb1-5) and RNA sequence analysis revealed that Gnb1 and Gnb2 are predominantly expressed in macrophages (Fig. 10A). Gnb1 and Gnb2 (schematically illustrated in Fig. 10B) have been implicated in chemotaxis in knockout studies using the mouse macrophage cell line J774A.1 (29,30). Recently, the Knockout Mouse Phenotyping Program (KOMP\textsuperscript{2}) (31) generated Gnb1 and Gnb2 knockout mice using CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein 9 (Cas9)). Homozygous deletion of Gnb1 was lethal, assessed at postnatal days 13-14 (Fig. 10C), consistent with the study by Okae and Iwakura (32) in which Gnb1 was disrupted by gene trap mutagenesis. Targeted deletion of Gnb2 was not lethal (Fig. 10D). The deletion allele, the location of primer binding sites, and an example of PCR genotyping are shown in Fig. 10E. In addition to the loss of exons 3-4, the 359 bp deletion mediated by guide RNAs and Cas9 introduces a frameshift and premature termination codon.

Gnb1\textsuperscript{-/-} macrophages exhibited robust lamellipodial membrane spreading in response to the application of complement C5a (Fig. 11A; supplemental Video S17). Moreover, the peaks of complement C5a-induced cell spreading and Ca\textsuperscript{2+} transients (examples are shown in the lower panels of Fig. 11A), indexed as Cal-520 fluorescence, were not significantly different...
Finally, we looked at the role of Gβ2-containing βγ-subunits (schematically illustrated in Fig. 12A) in complement C5a-mediated chemotaxis. Compared to WT macrophages migrating in a complement C5a gradient, Gnb2-deficient macrophages showed reduced velocity and impaired chemotactic navigation (Fig. 12B). Gnb2−/− macrophages polarized in the presence of complement C5a (Fig. 12C), but lamellipodial membrane protrusions were less efficiently directed towards higher concentrations of complement C5a (supplemental Videos S18 and S19). Summary data are plotted in Fig. 12D. Both cell velocity and chemotactic efficiency were significantly decreased in Gnb2−/− macrophages compared to WT controls.

Discussion

Intravital imaging, dating back to 1888, provides insight into the behavior of immune cells in their natural environment, but using this approach it has been difficult to study the chemotactic response of cells to a specific molecule. This problem was solved by the introduction of the Boyden chamber (33), consisting of two reservoirs separated by a thin membrane. However, in Boyden-type transwell assays the cells only move across a thin membrane, typically with a thickness of about 10 µm, making it difficult to assess cell morphology and to distinguish chemokinesis from chemotaxis. Using knockout mouse models and real-time chemotaxis assays, which allow visualization of cell morphology and measurement of cell velocity and chemotactic efficiency, we investigated the roles of specific G protein subunits (Gα2, Gα3, Gαi1, Gαi2/Gαi3, Gαi5, Gβi, and Gβ2) in transducing complement C5a gradients to directed migration. The specific inhibitor of Gαi0 subunits pertussis toxin abrogated complement C5a-mediated chemokinesis and chemotaxis, which was largely recapitulated in Gna12 (Gα2) knockout macrophages, whereas Gna13 (Gα3) mutants exhibited robust chemotactic efficiency, but marginally reduced cell velocity. Thus, Gα2 is a cornerstone of complement C5a-mediated chemotaxis, schematically illustrated in Fig. 13. However, Gna12−/− macrophages, as well as wild-type macrophages treated with pertussis toxin, still induced robust Ca2+ transients and lamellipodial membrane protrusions upon stimulation with complement C5a. Thus, Gα2 is not essential for the generation of membrane protrusions, but it is indispensable for the spatial and temporal regulation Rho GTPases and the biasing of protrusions towards higher complement C5a concentrations.

The role of Ca2+ transients in chemotaxis is controversial (see Artemenko et al. (3)). We found that complement C5a-mediated lamellipodial membrane protrusions were not impaired by sequestration of intracellular Ca2+. Moreover, we confirmed that C5aR-Gα15 signaling, which activates PLC-β, almost completely accounts for complement C5a-induced Ca2+ signaling, although both lamellipodial cell spreading and chemotaxis were intact in macrophages lacking Gα15. Similar to complement C5a, UTP, an endogenous P2Y2R ligand, induced large Ca2+ transients and lamellipodial membrane protrusions. However, we found that P2Y3R-induced Ca2+ transients strictly required Gαi2/Gαi1 and not Gαi5. Notably, ATP and UTP are poor long-range chemoattractants for macrophages and neutrophils (15,34,35), possibly due to lack of activation of Gα12 or other Gαi family members by P2Y3Rs and/or rapid degradation of the ligands by ectonucleotidases, such as CD39 and CD73 (36). Although we previously found that a stable (slowly hydrolyzed) analogue of ATP had no chemotactic activity for mouse macrophages (34), Collins et al. (35) observed that PLB-985 cells, a human neutrophil-like cell line, migrated transiently (for about 5 min) towards higher concentrations of ATP in a radial gradient generated by ultraviolet light-induced uncaging of caged-ATP. Thus, ATP may transiently evoke directed migration, whereas complement C5a acts as a durable chemoattractant. We have previously shown that complement C5a induces ATP release, which probably acts as a positive feedback loop since the presence of potato apyrase, which completely degraded ATP, ADP, and adenosine, inhibited complement C5a-mediated chemotaxis (16). Similarly, apyrase was shown to inhibit neutrophil chemotaxis to the chemoattractant fMLP, a formylated tripeptide (15).

C5aR-Gα15 signaling, as well as ATP/UTP-P2Y2R signaling, probably induce lamellipodial membrane protrusions independent of Gαi0 family subunits via activation of PLC-β isoforms and depletion of PIP2, as illustrated in Fig. 13. Depletion of PIP2 may cause dissociation of Rac-GAPs and Cdc42-GAPs from the membrane, along the lines described by Li et al. (37), thereby promoting increased activity of the Rac
subfamily of Rho GTPases, especially Rac1 and Rac2, and Cdc42.

We found that stimulation of wild-type macrophages with complement C5a increases the levels of active RhoA. However, we did not test whether this effect is lost in Gna12/Gna13 dKO macrophages, and therefore further experiments are required to determine whether complement C5a receptors couple to Gna12/Gna13. In any case, Gα12/Gα13 signaling does not appear to play a critical role in complement C5a-mediated chemotaxis. Macrophages lacking Gα12/Gα13 exhibited intact chemotaxis, but increased cell velocity and modestly impaired tail retraction. This observation fits in with our previous findings that Rhoa/Rhob (RhoA/RhoB) dKO macrophages exhibit the same behavior (intact complement C5a-mediated chemotaxis and increased velocity), except Rhoa/Rhob dKO cells develop much more markedly elongated trailing ends (25). Likewise, human monocytes treated with inhibitors of Rho or its major downstream effector Rho kinase exhibit both normal chemotaxis and elongated trailing ends when migrating on a 2D surface (38). Contrariwise, when Rho activity is increased, as in the case of macrophages from mice lacking the RhoGAP Myo9b (21), chemotaxis is impaired and cell velocity is decreased. The unconventional myosin Myo9b contains an atypical C1 domain (which does not bind to diacylglycerol) (39) and a RhoGAP domain, but not a PH domain, and probably acts as an actin-binding, motorized inhibitor of Rho at the front of migrating macrophages.

Using lentiviral delivery of siRNA into J774A.1 cells (mouse macrophage cell line), Hwang et al. (30) found that deletion of both Gβ1 and Gβ2 eliminated G protein-mediated receptor signaling by all four Gα families. Interestingly, in an earlier study, the authors showed that knockdown of Gβ2 alone in J774A.1 cells completely inhibited migration towards complement C5a in transwell assays, and cells showed absent or weak Ca2+ responses to complement C5a (29). The authors also found that PTX treatment prevented Ca2+ responses. In contrast, we found that complement C5a-induced Ca2+ signaling was intact in Gnb2−/− macrophages and only modestly reduced in WT cells treated with PTX, whereas Gna15−/− macrophages exhibited mostly absent or weak Ca2+ responses. However, we observed moderately impaired chemotaxis by Gnb2−/− macrophages in a complement C5a gradient. Presumably, Gβ1 partially compensates for loss of Gβ2 in Gnb2−/− macrophages. Further studies using macrophages from mice with myeloid-restricted deletion of Gnb1 would be helpful to explore the relative functions of Gβ1 and Gβ2 in complement C5a-mediated chemotaxis.

In summary, using mouse macrophages lacking specific G protein subunits and real-time imaging assays, we found that (i) the α-subunit Gα2 is indispensable, and Gα3 is largely redundant, for complement C5a-mediated chemotaxis, (ii) Gα13 mediates complement C5a-induced Ca2+ release, but is dispensable for chemotaxis, (iii) the Gα12/Gα13 family contributes to tail retraction, but is not required for chemotactic navigation, (iv) Gαq/Gα11 clearly mediate UTP- (and ATP-) induced Ca2+ release, but autocrine P2Y2-R–Gαq/Gα11 signaling is not important for complement C5a-induced Ca2+ release and chemotaxis, (v) deletion of Gβ1 is lethal, (vi) mice lacking Gβ2 are viable, (vii) Gβ2 is important for complement C5a-induced chemotaxis, but not Ca2+ signaling, and (viii) complement C5a-induced lamellipodial membrane protrusions persist in the absence of Gα2, Gα3, Gαq/Gα11, Gα12/Gα13, Gα15, or Gβ2.

Experimental procedures

Mice

Gnb1 knockout (Gnb1em1(IMPC)Bay) mice were generated by the Knockout Mouse Phenotyping Program (KOMP2) (31) at the Department of Molecular and Human Genetics (Baylor College of Medicine, Houston, TX) using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) gene targeting technique. Gnb2 knockout (C57BL/6NJ-Gnb2em1(IMPC)/Mmjax) mice were generated by KOMP2 at The Jackson Laboratory (Bar Harbor, ME) using CRISPR-Cas9. The knockout allele was produced by injecting Cas9 mRNA and four single guide RNAs (TCCCATTTCTCAGTGGCCCA, ATGGGCGAGATAATGATACA, TCCCCATTTCTCAGTGCCCA and ATGATGGGCACTGCAAGAGA) into C57BL/6NJ-derived fertilized eggs, which were subsequently transferred to pseudopregnant females. The guide RNAs, in combination with the (RNA-guided) DNA endonuclease Cas9, were designed to delete 359 bp in the targeted gene (Gnb2), leading to a deletion including exons 3-4 and a frameshift after amino acid 19. The frameshift produces a stop codon after coding a further 19 residues. Pups were genotyped using the primer pair (sequences 5′→3′) Gnb2-F (CCCAAATCTCTCAGGATGA)
and Gna2-R (TGCTTCCCTTTGACCTGAGT), which resulted in a 639 bp PCR product for the WT allele and a 280 bp product for the mutant allele. The generation of Gna12 (40) and Gna13 (41) knockout mice, which were backcrossed onto a C57BL/6J genetic background (13), have been previously described. Gna12<sup>−/−</sup> and Gna13<sup>−/−</sup> mice were kindly provided by Johannes Engelbert Gessner (Hannover, Germany). The production of double transgenic mice with floxed (fl) Gnaq alleles (Gnaq<sup>fl/fl</sup>) and homozygous deletion (−/−) of Gna11 (Gna11<sup>−/−</sup>) has been reported by Wettenschureck et al. (42). Myeloid-restricted Gnaq/Gna11 double knockout (Gnaq<sup>fl/fl</sup>/LysM-Cre/Gna11<sup>−/−</sup>) mice were derived by crossing B. Braun, Melsungen, Germany) using 2 x 4.5 ml ice-cold Hank’s balanced salt solution (HBSS) without Ca<sup>2+</sup> or Mg<sup>2+</sup> (14175-046; Sigma-Aldrich, Steinheim, Germany), which had been filtered using a 0.2 µm cellulose acetate membrane (723-2520; Thermo Fisher Scientific). Aliquots were stored at −80 °C. Myeloid-restricted Gna12/Gna13 double knockout (Gna12<sup>−/−</sup>/Gna13<sup>fl/fl</sup>/LysM-Cre) mice. All animal experiments were performed in accordance with the German Animal Welfare Act (Tierschutzgesetz), approved by the local ethics committee of the University of Münster, and conformed to the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health.

**Isolation of resident peritoneal macrophages**

The method, including a video, for isolation of mouse resident peritoneal macrophages has recently been described (45). Mice were killed by an overdose of isoflurane in air, and the peritoneal cavity was lavaged via a 24-G plastic catheter (B. Braun, Melsungen, Germany) using 2 x 4.5 ml ice-cold Hank’s balanced salt solution (HBSS) without Ca<sup>2+</sup> or Mg<sup>2+</sup> (14175-046; Gibco, Life Technologies, Grand Island, NY), after centrifugation (300 x g for 6.5 min), cells were resuspended in bicarbonate-free RPMI 1640 medium containing 20 mM Hepes, which was supplemented with 10 % heat-inactivated FCS, 100 ng/ml lipopolysaccharide, and antibiotics. Next, 15 µl medium containing chemoattractant (complement C5a) and 0.003 % Patent Blue V (blue dye) was drawn into one of the reservoirs. The final concentration of complement C5a was 20 nM. Complement C5a (2150-C5-025; R&D Systems, Abingdon, United Kingdom) was reconstituted in phosphate buffered saline containing 0.1 % bovine serum albumin which had been filtered using a 0.2 µm cellulose acetate membrane (723-2520; Thermo Fisher Scientific). Aliquots were stored at −80 °C. An aluminium heating block maintained at 37 °C was used to keep media and the µ-Slide Chemotaxis chamber warm during the filling procedure. The observation area was imaged by phase-contrast microscopy via a 10×/0.3 objective lens. The blue dye served as a visual indicator of gradient formation. Images were captured every 2 min for 14 h, and cell migration tracks between 6 h and 12 h were analyzed with ImageJ (National Institutes of Health) using a cell tracking plugin and the chemotaxis and migration tool from Ibidi. Twenty-five cells which remained in the field of view were tracked for each chemotaxis assay.

**Time-lapse imaging of intracellular [Ca<sup>2+</sup>] and lamellipodial membrane dynamics**

After overnight incubation of resident peritoneal cells, seeded in fibronectin-coated µ-Slide I chambers, in modified RPMI 1640 medium containing sodium bicarbonate, the medium was switched to bicarbonate-free RPMI 1640 medium containing 20 mM Hepes, 1 mM N-(2-
intracellular anionic fluorescent Ca\(^{2+}\) indicators transporters which reduces the efflux of a competitive blocker of organic anion sodium salt (P36400; Thermo Fisher Scientific), 200 mM stock solution of the water soluble and additionally 1 mM probenecid, added from a containing 1 mM MPG, 10 % FCS, antibiotics, diluted 1:1000 in RPMI 1640- Hepes medium the 10 mM Cal -520/AM stock solution was 4.53 µl of a 20 % solution of Pluronic F-127 (Sigma-Aldrich) was prepared by adding 4.53 µl of a 20 % solution of Pluronic F-127 in dimethyl sulfoxide to a 50 µg aliquot of Cal-520/AM. To prepare a 10 µM loading solution, dimethyl sulfoxide to a 50 µg aliquot of Cal-520/AM. To prepare a 10 µM loading solution, the 10 mM Cal-520/AM stock solution was diluted 1:1000 in RPMI 1640-Hepes medium containing 1 mM MPG, 10 % FCS, antibiotics, and additionally 1 mM probenecid, added from a 200 mM stock solution of the water soluble sodium salt (P36400; Thermo Fisher Scientific), a competitive blocker of organic anion transporters which reduces the efflux of intracellular anionic fluorescent Ca\(^{2+}\) indicators (46). Cells were loaded with Cal-520 by 20 min incubation with 10 µM Cal-520/AM at 37 °C and subsequently washed with RPMI 1640-Hepes medium containing MPG and probenecid. Lamellipodial membrane dynamics were imaged by either brightfield or fluorescence microscopy. In the latter case, the plasma membrane was stained with CellMask Orange (C10045; Thermo Fisher Scientific), diluted 1:1000 (from a 5 mg/ml stock solution in dimethyl sulfoxide) in modified RPMI 1640-Hepes medium or in the above Cal-520 loading solution. Cells were incubated for 10-20 min at 37 °C.

Cells were imaged via the Apochromat TIRF 60x/1.49 (oil-immersion) objective lens of a Nikon Eclipse Tii inverted microscope, which was connected to a spinning disk confocal system (UltraVIEW Vox 3D live cell imaging system). The system included a Yokogawa (Japan) CSU-X1 spinning disk scanner, a Hamamatsu (Japan) C9100-50 EM-CCD camera (1000 × 1000 pixels), and Volocity software. Cal-520 was excited with a 488 nm laser, whereas CellMask Orange was excited via a 561 nm laser. Images were captured (at a fixed focus level) using 2×2 binning (giving 500 × 500 pixels per image) at a rate of 2 s per timepoint. Alternatively, cells were alternately imaged by brightfield and fluorescence microscopy at a slower rate of 5 s per timepoint. Focus drift during long recordings was prevented using the Nikon Perfect Focus System, which maintains the position of the coverslip in the z-axis by reflecting near-infrared light (870 nm) and detecting it via a CCD (charge-coupled device) line sensor. The temperature was maintained at 37 °C using an Okolab all-in-one stage incubator (Okolab, Ottaviano, Italy) and an objective lens heating mantle (Scientific Instruments, Farmingdale, NY).

Flow cytometry and cell sorting
Freshly isolated mouse resident peritoneal cells were stained with Alexa Fluor 488-conjugated anti-F4/80 antibodies and resuspended in autoMACS running buffer (Miltenyi Biotec, Bergisch Gladbach, Germany), which contains phosphate buffered saline, 2 mM EDTA, and 0.5 % bovine serum albumin. Purification of F4/80\(^+\) cells (mouse macrophages) was performed using a BD FACS Aria II (or FACS Aria III) cell sorter (BD Biosciences, San Jose, CA). Recovered cells were centrifuged at 300 × g for 5 min and the supernatant was removed before proceeding to RNA isolation.

RNA isolation and RNA-Seq analysis
Total RNA of purified mouse resident peritoneal F4/80\(^+\) cells (macrophages) was isolated by solid phase extraction using a Direct-zol\textsuperscript{TM} RNA MicroPrep kit (Zymo Research, Freiburg, Germany) according to the manufacturer’s instructions. Briefly, we lysed the pelleted cells with 300 µl TRIzol (Thermo Fisher Scientific), added 300 µl analytical (100 %) ethanol, and transferred the mixture into a Zymo-Spin\textsuperscript{TM} IC column inserted into a collection tube. Following wash steps, as well as DNase treatment for 15 min, involving several centrifugations at 12,000 × g, purified total RNA was captured in a silica column. Using RNase-free water, concentrated RNA was eluted from the silica column and collected in a DNase-/RNase-free safe-lock tube.
Isolated RNA samples were tested for integrity using RNA ScreenTape (Agilent Technologies, Santa Clara, CA) and stored at -80 °C. Next-generation sequencing was performed using a NextSeq 500 Sequencing System (Illumina, San Diego, CA). Samples were prepared using a TruSeq xRNA sample preparation kit (Illumina), which involved the steps: purification and fragmentation of mRNA, first and second strand cDNA synthesis, end repair, adenylation of 3’ ends, ligation with adaptors, and PCR amplification. RNA-Seq data were analyzed using the Tuxedo suite, an open access set of applications for ultrafast alignment of short reads to the genome, recognition of splice junctions, and differential expression analysis. G-LISA assay for active RhoA

Levels of active RhoA (RhoA-GTP) were measured using a colorimetric RhoA G-LISA Activation Assay Kit (Cat. # BK124; Cytoskeleton, Denver, CO), according to the elaborate instruction manual. The microplate was shaken at a speed of 400 revolutions per minute using Fisherbrand™ microplate shakers (15504070; Fisher Scientific), one of which was placed in a cool room (4 °C). Mouse bone marrow-derived macrophages were used instead of peritoneal macrophages to provide sufficient lysates for the assays. Femurs from each mouse were fractured with a scalpel blade at the mid-diaphysis level and bone marrow cells were flushed out of each fragment using 5 ml RPMI 1640-Hepes medium containing 10 % heat-inactivated FCS and antibiotics, which was supplemented with non-essential amino acids and 15 ng/ml recombinant mouse macrophage colony-stimulating factor (416-ML-010; R&D Systems). The suspension was pipetted into a Teflon bag (PermaLife PL30, OriGen, Biomed Europe), such that each Teflon bag contained cells from a single mouse, and incubated at 37 °C with 5 % CO₂ for 6 days. The Teflon bag was placed in ice for 30 min and the cells, resuspended by shaking and massaging, were poured into a 50 ml tube. The tube was centrifuged at 300 × g for 10 min and the pellet was resuspended in RPMI 1640-Hepes medium containing 10 % heat-inactivated FCS, and antibiotics, and seeded into 35 mm round culture dishes. After 1.5 h incubation at 37 °C in a CO₂-free incubator, the medium was switched to RPMI 1640 medium containing sodium bicarbonate, 10 % heat-inactivated FCS, and antibiotics, and incubated overnight at 37 °C with 5 % CO₂. Stimulation of cells and the harvesting of lysates were performed after switching back to Hepes-containing medium and allowing at least 1 h equilibration time.

Statistics

The non-parametric Mann-Whitney U test or Kruskal-Wallis one-way analysis of variance on ranks was used to test for statistical differences at the 0.05 level of significance. Post-hoc multiple comparisons were made using the Bonferroni correction. Statistical analyses were performed using Origin 2020 (OriginLab) or earlier versions, and data are presented as box plots or mean ± standard error (s.e.m.), unless stated otherwise.

Data availability

All data are contained within the manuscript.
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Author contributions
N.W., G.I., T.M.W., and P.J.H. generated resources and/or designed experiments. E.v.d.B., B.A., M.H., S.W., A.C.B., and P.J.H. performed research and analyzed data. P.J.H. wrote the paper.

Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

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Figure 1. Expression of Gα subunits in macrophages and roles of Gnai2 and Gnai3 in complement C5a-mediated chemotaxis. A, expression levels of Gα subunits in mouse resident peritoneal F4/80+ cells (macrophages). RNA-Seq analysis was performed using RNA isolated from resident peritoneal F4/80+ cells purified by cell sorting (n = 3 mice). The inset, superimposed graph with an interrupted y-axis, shows the expression levels of receptors for complement components 3a and 5a. B, schematic diagram showing a complement C5a receptor (C5aR), a member of the G protein-coupled receptor superfamily, and a heterotrimeric G protein in which the subunits are color coded blue (Gαi), green (Gβ), and white (Gγ). The four Gα families (Gαi, Gαo, Gαq, and Gα12) are listed below the blue α-subunit (Gα) together with the corresponding genes investigated with knockout mouse models, including two genes encoding β-subunits: Gnb1 (Gβ1) and Gnb2 (Gβ2). C, migration plots of wild-type (WT), pertussis toxin (PTX) treated WT, Gnai2-/-, and Gnai3-/- macrophages in a chemotactic complement C5a gradient. D, summary box plots of cell velocity and chemotactic efficiency (chemotaxis index), calculated by dividing the displacement along the y-axis by the cumulative distance migrated. The chemotaxis index is also known as the y-forward migration index and has a range of -1 to +1. *p < 0.05; Kruskal-Wallis test and post-hoc Mann-Whitney U test with Bonferroni correction (n = 75 for each group, except n = 50 for the WT+PTX group; 3 independent experiments). E, example (green trace) of a complement C5a-induced Ca2+ transient measured in the absence and presence of EGTA/AM. *p < 0.05 (n.s. stands for not significant); Mann-Whitney U test (n = 50 (WT), n = 19 (WT+PTX), n = 46 (WT+EGTA/AM); n = 3 independent experiments). F, representative example, from 2 independent experiments, of RhoA activity measured using a colorimetric G-LISA assay, in which active RhoA (RhoA-GTP) was indexed as absorbance at 450 nm, migration plots of wild-type (WT), pertussis toxin (PTX) treated WT, Gnai2-/-, and Gnai3-/- macrophages in a chemotactic complement C5a gradient. *p < 0.05; Kruskal-Wallis test and post-hoc Mann-Whitney U test with Bonferroni correction (n = 50 cells per group; sampled from 2 independent experiments). 

Figure 2. Complement C5a-induced Ca2+ transients and lamellipodial membrane protrusions are not impaired in Gnai2-/- or Gnai3-/- macrophages. A, simultaneous imaging of intracellular [Ca2+] (green trace) and projected cell area (black trace) in individual wild-type (WT) and Gnai2-/- macrophages challenged with 20 nM complement C5a. Intracellular [Ca2+] is indexed as relative Cal-510 fluorescence intensity (F0/F) in which the measured fluorescence intensity (F) is divided by the resting fluorescence intensity (F0) after subtracting the background fluorescence intensity at each timepoint. B, summary box plots of peak complement C5a-induced Ca2+ transients and projected cell area. *p < 0.05 (n.s. stands for not significant); Kruskal-Wallis test and post-hoc Mann-Whitney U test with Bonferroni correction (n = 50 (WT), n = 19 (WT+PTX), n = 46 (Gnai2-/-) and n = 9 (Gnai3-/-); 2-3 independent experiments). 

Figure 3. Sequestration of intracellular [Ca2+] with EGTA does not prevent complement C5a-induced lamellipodial membrane protrusions. A, example (green trace) of a complement C5a-induced Ca2+ transient largely blocked in a wild-type (WT) macrophage after passively loading the cell with the Ca2+ chelator EGTA using its acetoxymethyl (AM) ester form (EGTA/AM). The box plots on the right show peak complement C5a-induced Ca2+ transients measured in the absence and presence of EGTA/AM. B, the trace shows the projected cell area corresponding to the above Ca2+ trace (panel A). The box plots on the right show peak complement C5a-induced cell spreading in the absence and presence of EGTA/AM. *p < 0.05 (n.s. stands for not significant); Mann-Whitney U test (n = 50 (WT pool) and n = 43 (WT+EGTA/AM)); n = 3 independent experiments). 

Figure 4. Complement C5a-mediated chemotaxis is preserved in Gnaq/Gna11 double knockout and Gna12/Gna13 double knockout macrophages. A, schematic diagram highlighting genes of the Gaα/Ga11 (Gnaq and Gna11) and Ga12/Ga13 (Gna12 and Gna13) families of Ga subunits which potentially may be activated by the complement C5a receptor (C5aR). B, summary box plots of cell velocity and chemotactic efficiency (chemotaxis index). *p < 0.05; Kruskal-Wallis test and post-hoc Mann-Whitney U test with Bonferroni correction (n = 75 for each group; 3 independent experiments). C, migration plots of wild-type (WT), Gnaq/Gna11 double knockout (dKO), and Gna12/Gna13 dKO macrophages in a chemotactic complement C5a gradient. D, 200 μm × 300 μm snapshots of WT, Gnaq/Gna11 dKO, and Gna12/Gna13 dKO macrophages in a chemotactic complement C5a gradient. Black arrows indicate elongated trailing ends. The schematic diagram on the left shows a µ-Slide Chemotaxis chamber with one of the two 40 μl reservoirs (filled with a blue dotted pattern) containing 20 nM complement C5a. E, box plots of maximal tail lengths developed by macrophages migrating in a chemotactic complement C5a gradient over a 6 h period. *p < 0.05; Kruskal-Wallis test and post-hoc Mann-Whitney U test with Bonferroni correction (n = 50 cells per group; sampled from 2 independent experiments).
Figure 5. UTP- and complement C5a-induced Ca\(^{2+}\) transients in Gnaq/Gna11 double knockout macrophages. A, time-lapse images (90 \(\mu\)m \(\times\) 90 \(\mu\)m) of wild-type (WT) macrophages loaded with the fluorescent Ca\(^{2+}\) indicator Cal-520. Uridine 5\(^{\prime}\)-triphosphate (UTP) was added as indicated. Scale bar, 10 \(\mu\)m. The intracellular Ca\(^{2+}\) signaling corresponding to the labeled macrophage (M\(\Phi\)1) is shown below. Intracellular [Ca\(^{2+}\)] is indexed as relative Cal-520 fluorescence intensity (F/F\(0\)), where the measured fluorescence intensity (F) is divided by the resting fluorescence intensity (F\(0\)) after subtracting the background fluorescence intensity at each timepoint. B, time-lapse images (90 \(\mu\)m \(\times\) 90 \(\mu\)m) of Gnaq/Gna11 double knockout (dKO) macrophages loaded with the fluorescent Ca\(^{2+}\) indicator Cal-520. UTP was added as indicated, and 22 min later complement C5a was applied to the same cells. Scale bars, 10 \(\mu\)m. The intracellular Ca\(^{2+}\) signals corresponding to the labeled macrophages (M\(\Phi\)1, M\(\Phi\)2 and M\(\Phi\)3) are shown below.

Figure 6. Intact complement C5a-induced Ca\(^{2+}\) transients in Gna12/Gna13 double knockout and Gnaq/Gna11 double knockout macrophages. A, time-lapse images (90 \(\mu\)m \(\times\) 90 \(\mu\)m) of wild-type (WT), Gnaq/Gna11 double knockout (dKO) and Gna12/Gna13 dKO macrophages loaded with the fluorescent Ca\(^{2+}\) indicator Cal-520. Complement C5a was added as indicated. Scale bars, 10 \(\mu\)m. B, intracellular Ca\(^{2+}\) signals corresponding to the above labeled macrophages (M\(\Phi\)s; panel A). Intracellular [Ca\(^{2+}\)] is indexed as relative Cal-520 fluorescence intensity (F/F\(0\)), where the measured fluorescence intensity (F) is divided by the resting fluorescence intensity (F\(0\)) after subtracting the background fluorescence intensity at each timepoint. C, summary peak [Ca\(^{2+}\)] data. n.s. = not significant; Kruskal-Wallis test (n = 20-27 per group; 2-3 independent experiments).

Figure 7. Gna15 is redundant for complement C5a-mediated chemotaxis. A, schematic diagram highlighting that Gna15 belongs to the Ga\(_{q}/Ga_{11}\) family of \(\alpha\)-subunits. B, migration plots of wild-type (WT) and Gna15\(^{-/-}\) macrophages in a chemotactic complement C5a gradient. C, 200 \(\mu\)m \(\times\) 300 \(\mu\)m snapshot of WT and Gna15\(^{-/-}\) macrophages in a chemotactic complement C5a gradient. D, summary box plots of cell velocity and chemotactic efficiency (chemotaxis index), calculated by dividing the displacement along the y-axis by the cumulative distance migrated. n.s. = not significant; Mann-Whitney U test (n = 75 per group; 3 independent experiments).

Figure 8. Complement C5a-induced Ca\(^{2+}\) transients are largely abolished in Gna15-deficient macrophages. A, time-lapse images (90 \(\mu\)m \(\times\) 90 \(\mu\)m) of wild-type (WT) macrophages loaded with the fluorescent Ca\(^{2+}\) indicator Cal-520. Complement C5a and uridine 5\(^{\prime}\)-triphosphate (UTP) were added as indicated. Scale bar, 10 \(\mu\)m. Below the series of four images is the intracellular Ca\(^{2+}\) signaling corresponding to the macrophage (M\(\Phi\)) labeled M\(\Phi\)1. Intracellular [Ca\(^{2+}\)] is indexed as relative Cal-520 fluorescence intensity (F/F\(0\)), where the measured fluorescence intensity (F) is divided by the resting fluorescence intensity (F\(0\)) after subtracting the background fluorescence intensity at each timepoint. B, time-lapse images (90 \(\mu\)m \(\times\) 90 \(\mu\)m) of Gna15\(^{-/-}\) macrophages loaded with Cal-520. Complement C5a and UTP were added as indicated. Scale bar, 10 \(\mu\)m. Below are traces corresponding to the labeled Gna15\(^{-/-}\) macrophages (M\(\Phi\)1 and M\(\Phi\)2, respectively). C, summary peak [Ca\(^{2+}\)] data. *p < 0.05; Mann-Whitney U test (n = 14 for WT (2 independent experiments); n = 30 for Gna15\(^{-/-}\) (3 independent experiments)).

Figure 9. Intact complement C5a-induced lamellipodial membrane spreading and Ca\(^{2+}\) transients in Gna15\(^{-/-}\) macrophages. A, time-lapse images (90 \(\mu\)m \(\times\) 90 \(\mu\)m) of wild-type (WT) and Gna15\(^{-/-}\) macrophages stained with the fluorescent plasma membrane marker CellMask Orange and loaded with the fluorescent Ca\(^{2+}\) indicator Cal-520. Complement C5a was added as indicated. The white arrows indicate examples of lamellipodial membrane protrusion. Scale bars, 10 \(\mu\)m. Below is the intracellular Ca\(^{2+}\) signaling corresponding to the Gna15\(^{-/-}\) macrophage labeled M\(\Phi\)1. Complement C5a and uridine 5\(^{\prime}\)-triphosphate (UTP) were added as indicated. Intracellular [Ca\(^{2+}\)] is indexed as relative Cal-520 fluorescence intensity (F/F\(0\)), where the measured fluorescence intensity (F) is divided by the resting fluorescence intensity (F\(0\)) after subtracting the background fluorescence intensity at each timepoint. B, box plots of projected cell area before and after application of complement C5a to WT or Gna15\(^{-/-}\).
macrophages. *p < 0.05; Mann-Whitney U test. C, box plots of relative peak projected cell area after application of ligand-free medium (sham) and complement C5a-containing medium to WT and Gna15+/− macrophages. *p < 0.05; Mann-Whitney U test (n = 34 for WT and n = 29 for Gna15+/− (3 independent experiments)). D, box plots of the changes in cell area (pre-stimulation cell area subtracted from the peak post-stimulation cell area) after stimulating WT and Gna15+/− macrophages with complement C5a (n.s. = not significant; Mann-Whitney U test).

Figure 10. Homozygous deletion of Gnb1 is lethal, whereas Gnb2-deficient mice are viable. A, expression levels of Gβ and Gγ subunits in mouse resident peritoneal F4/80+ cells (macrophages). RNA-Seq analysis was performed using RNA isolated from resident peritoneal F4/80+ cells purified by cell sorting (n = 3 mice). B, schematic diagram highlighting the genes encoding the Gβ-subunits Gβ1 (Gnb1) and Gβ2 (Gnb2). C, homozygous deletion of Gnb1 is lethal. D, homozygous Gnb2 mutant mice are viable. E, schematic diagram of the WT allele and the Gnb2 knockout allele, generated by CRISPR/Cas9. The forward (Gnb2-F) and reverse (Gnb2-R) primers are indicated by horizontal red arrow heads, where the arrow tip marks the start of binding. Below is an image of an agarose gel depicting the genotyping.

Figure 11. Gnb2−/− macrophages show robust complement C5a-induced cell spreading and Ca2+ transients. A, time-lapse images (90 µm × 90 µm) of wild-type (WT) and Gnb2−/−macrophages stained with the fluorescent plasma membrane marker CellMask Orange and loaded with the fluorescent Ca2+ indicator Cal-520. Complement C5a was added as indicated. The white arrows indicate examples of lamelliodal membrane protrusion. Scale bars, 10 µm. Below the series of cell morphology (CellMask Orange) images is the intracellular Ca2+ signaling corresponding to the individual macrophages labeled MF1. Complement C5a and uridine 5′-triphosphate (UTP) were added as indicated. Intracellular [Ca2+] is indexed as relative Cal-520 fluorescence intensity (F/F0), where the measured fluorescence intensity (F) is divided by the resting fluorescence intensity (F0) after subtracting the background fluorescence intensity at each timepoint. B, summary box plots of peak cell spreading and peak intracellular [Ca2+] induced by complement C5a. n.s. = not significant; Mann-Whitney U test (n = 52 per group; 3 independent experiments).

Figure 12. Gnb2−/− macrophages have decreased velocity and impaired navigation in a chemotactic complement C5a gradient. A, schematic diagram highlighting the Gβ-subunit Gβ2 (Gnb2). B, migration plots of wild-type (WT) and Gnb2−/−macrophages in a chemotactic complement C5a gradient. C, 200 µm × 300 µm snapshot of WT and Gnb2−/−macrophages in a chemotactic complement C5a gradient. D, summary box plots of cell velocity and chemotactic efficiency (chemotaxis index), calculated by dividing the displacement along the y-axis by the cumulative distance migrated. *p < 0.05; Mann-Whitney U test (n = 75 per group; 3 independent experiments).

Figure 13. Tabular summary and schematic diagram of G protein subunits involved in transducing complement C5a gradients into directed migration. A, tabular summary of results. B, schematic summary. Complement C5a receptors (C5aRs) couple (i) directly to at least two heterotrimeric G proteins formed by Ga15 and Ga12 subunits, and possibly also Ga12/Ga13 and Ga13 (not shown) subunits, and their respective Gβγ subunits and (ii) indirectly to Gaq/Gα11-containing heterotrimeric G proteins via autocrine ATP signaling, which stimulates P2Y2 receptors (P2Y2Rs). The Ga12 subunit is indispensable for chemotaxis and associates with Gβ2-containing, or possibly also Gβ1-containing, Gβγ subunits. Ga12/Gβ2γx heterotrimeric G proteins, where X is unknown, dissociate into active (GTP-bound) Ga12 subunits and Gβ2γx dimers following receptor activation by complement C5a. The Gβ2γ3 (or possibly Gβ1γ3) dimers activate phosphoinositide 3-kinases (PI3Ks), which catalyze the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 is known to recruit pleckstrin homology domain-containing Rac- and Cdc42-guanine nucleotide exchange factors (GEFs) to the membrane. Activation of Ga15-containing heterotrimeric G proteins directly by complement C5a, as well as indirect activation of Gaq/Gα11-containing heterotrimeric G proteins via autocrine ATP and UTP signaling, increases the activity of phospholipase C-β (PLC-β) isoforms, which catalyze the hydrolysis of PIP3 to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces Ca2+ release from the endoplasmic reticulum, but this Ca2+ signal is largely redundant for lamellipodial membrane protrusions and chemotaxis.
However, we speculate that depletion of PIP$_2$ by PLC-β isoforms and PI3Ks contributes to the formation of lamellipodial membrane protrusions by promoting the dissociation of Rac- and Cdc42-GTPase-activating proteins (GAPs). We speculate that activation of Ga$_{12}$/Ga$_{13}$ by complement C5a-C5aR signaling, which remains to be confirmed, increases the activity of the monomeric (small) G proteins RhoA and RhoB via RhoGEFs. Activated (GTP-bound) RhoA and RhoB promote actomyosin-independent retraction of the trailing end of migrating cells, while the RhoGAP Myo9b is thought to inhibit RhoA and RhoB at the front of cells. Extracellular ATP and UTP stimulate P2Y$_2$Rs. ATP, but not UTP, additionally activates P2X receptors (not shown), ligand-gated cation channels. ATP and UTP are rapidly degraded by surface ectonucleotidases, such as CD39, to form ligands for other purinergic receptors (not shown).
**Figure 1**

(A) Gene expression (read counts) for different G-protein alpha subunits.

(B) Diagram of the Complement C5a receptor (C5a-R) with interactions involving various G-protein alpha subunits (Gαi2, Gαi3, Gαs, Gβ/γ, Gαq, Gα11, Gα15).

(C) Diagrams showing the movement of F4/80+ cells (macrophages) under different conditions:
- **WT**
- **WT + PTX**
- **C5a**
- **C5a + Gαi2**
- **C5a + Gαi3**

(D) Box plots displaying velocity and chemotaxis index for different groups:
- **WT**
- **WT + PTX**
- **Gαi2**
- **Gαi3**

*Significant differences indicated by asterisks.*
Figure 2
Figure 3

**Figure 3A**
- WT + EGTA/AM
- 20 nM C5a
- Peak C5a-induced [Ca\(^{2+}\)]\(_i\) (F/F\(_0\))

**Figure 3B**
- Cell area (µm\(^2\))
- C5a-induced cell spreading (rel.)

- n.s.
- *
Figure 4
Figure 5

A

WT

t = -1 min

+100 µM UTP

MΦ 1

MΦ 2

MΦ 3

100 µM UTP

20 nM C5a

WT MΦ 1

B

MΦ 1

MΦ 2

MΦ 3

Gnaq/Gna11 dKO

t = -1 min

+100 µM UTP

100 µM UTP

100 µM UTP

+20 nM C5a

MΦ 1

MΦ 2

MΦ 3

20 nM C5a

20 nM C5a

20 nM C5a
Figure 6
Figure 7

A

Complement C5a

\[ \alpha \beta \gamma \]

Gnb1, Gnb2

Gna2, Gna3

Gs

Gq/11

Gna12, Gna13

Gna15

B

WT

C5a

Gna15−/−

C5a

C

WT

C5a

Gna15−/−

C5a

D

Velocity (µm/min)

Chemotaxis index

WT

Gna15−/−

WT

Gna15−/−

n.s.

n.s.

n.s.
Figure 8

A. WT MΦ

- t = -1 min
- +20 nM C5a
- t = +1 min
- +100 µM UTP

B. Gna15^-/- MΦ

- t = -1 min
- +20 nM C5a
- t = +1 min
- +100 µM UTP

C. Comparison of Cal-520 (F/F_0) levels between WT and Gna15^-/- MΦs

- +C5a
- +UTP (post-C5a)
Figure 9

A

CellMask Orange (plasma membrane stain)

WT

+20 nM C5a  t = +1 min  +2 min

Gna15−/−

+20 nM C5a  t = +1 min  +2 min

Mφ 1

B

C

D

n.s.

20 nM C5a  100 µM UTP

Gna15−/− Mφ 1

Cell area (µm²)

Pre-C5a +C5a  Pre-C5a +C5a

Gna15−/−

Cell spreading (rel.)

Sham +C5a Sham +C5a

Gna15−/−

Cell area change (µm²)

WT Gna15−/−
Figure 10

(A) Gene expression (read counts) for Gnb1, Gnb2, Gnb3, Gnb4, Gnb5, Gnb6, Gnb7, Gnb8, Gnb10, Gnb11, and Gnb12.

(B) Diagram of Complement C5a signaling with receptors and G proteins.

(C) Number of mice for Gnb1+/+ × Gnb1+/+ (postnatal) genotypes.

(D) Number of mice for Gnb2+/+ × Gnb2+/+ (postnatal) genotypes.

(E) Gel electrophoresis showing bands at 639 bp and 280 bp for Gnb2 genotypes.

Figure 10
Figure 11

CellMask Orange (plasma membrane stain)

WT MΦ 1

Gnb2/- MΦ 1

+20 nM C5a  t = +1 min  +2 min

20 nM C5a  100 µM UTP

Peak cell spreading (relative)

Peak C5a-induced Cal-520 signal (F/F0)

WT Gnb2/-

n.s.

Figure 11
Figure 12
**Figure 13**

### Table A

| Gene Deletion | C5a → Ca²⁺ | UTP → Ca²⁺ | C5a-induced spreading | Polarization | Cell velocity | Chemotaxis |
|---------------|------------|------------|------------------------|--------------|--------------|------------|
| Gna12/Gna13 dKO | ++         | ++         | ++                     | ++           | ++           | ++         |
| Gna15⁻/⁻ | ++         | ++         | ++                     | ++           | ++           | ++         |
| Gnai2⁻/⁻ | ++         | n.d.       | ++                     | ↓↓           | ↓↓           | ↓↓         |
| Gnai3⁻/⁻ | ++         | n.d.       | ++                     | ++           | ++           | ++         |
| Gnaq/Gna11 dKO | ++         | ↓↓         | ++                     | ++           | ++           | ++         |
| Gna15⁻/⁻ | ++         | ++         | ++                     | ++           | ++           | ++         |
| Gnb1⁻/⁻ (lethal) | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Gnb2⁻/⁻ | ++         | ++         | ++                     | ++           | ↓            | ↓          |

### Table B

- ADP/UDP → ATP/UTP
- Autocrine signaling
- Complement C5a gradient
- LPA (serum)

**Diagram B**

- P2Y₂ R → ATP/UTP → Autocrine signaling
- C5a R → RhoGEFs
- Gnaq/Gna11
- Gnai2, Gnb2
- Gna12/Gna13
- Gnai3
- Gnai11
- Gnb1
- Gnb2
- Myo9b

**Process**

- Ca²⁺ Recruitment of Rac-/Cdc42-GEFs at the front
- Lamellipodial protrusions and chemotaxis
- PM2
- PLC-β
- DAG + IP₃
- PIP₂ Depletion
- PIP₃
- RhoA/RhoB
- RhoGEFs
- Tail retraction
- Redundant for chemotactic navigation

**Legend**

- + + +
- ++
- ↓↓
- n.d.

**Note**

- C5a → Ca²⁺ C5a-induced spreading
- Polarization
- Cell velocity
- Chemotaxis

---

**Gene Deletion**

- Gna12/Gna13 dKO
- Gna15⁻/⁻
- Gnai2⁻/⁻
- Gnaq/Gna11 dKO
- Gnai3⁻/⁻
- Gnb1⁻/⁻ (lethal)
- Gnb2⁻/⁻
Knockout mouse models reveal the contributions of G protein subunits to complement C5a receptor-mediated chemotaxis

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