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$G\alpha_{i2}$ is required for chemokine-induced neutrophil arrest

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Abstract

Chemokines including CXCL1 participate in neutrophil recruitment by triggering the activation of integrins, which leads to arrest from rolling. The downstream signaling pathways which lead to integrin activation and neutophil arrest following G-protein coupled receptor engagement are incompletely understood. To test whether $G\alpha_{i2}$ is involved, mouse neutrophils in their native whole blood were investigated in mouse cremaster postcapillary venules and in flow chambers coated with P-selectin, ICAM-1 and CXCL1. *Gnai2*$^{-/-}$ neutrophils showed significantly reduced CXCL1-induced arrest *in vitro* and *in vivo*. Similar results were obtained with LTB_4. Lethally irradiated mice reconstituted with *Gnai2*$^{-/-}$ bone marrow showed a similar defect in chemoattractant-induced arrest as *Gnai2*$^{-/-}$ mice. In thioglycollate-induced peritonitis and LPS-induced lung inflammation, chimeric mice lacking $G\alpha_{i2}$ in hematopoietic cells showed about 50% reduced neutrophil recruitment similar to that seen in *Gnai2*$^{-/-}$ mice. These data show that neutrophil $G\alpha_{i2}$ is necessary for chemokine-induced arrest, which is relevant for neutrophil recruitment to sites of acute inflammation.

Introduction

Leukocyte recruitment to sites of inflammation proceeds in a multistep cascade beginning with capture and rolling, followed by arrest, adhesion strengthening, and transmigration. [1,2] This process occurs as a result of molecular changes on the surface of leukocytes and endothelial cells following inflammatory stimuli. During rolling, neutrophils integrate numerous activating signals [3] resulting in β_2-integrin activation, slow rolling and arrest. Activation of β_2-integrins is associated with a conformational change from a bent conformation to an extended conformation that supports slow rolling [4,5] and, ultimately, to a high affinity state that supports arrest. [6,7] Arrest chemokines are presented on the surface of endothelial cells and can cause arrest of rolling leukocytes. The interaction of CXCL1 (formerly known as keratinocyte-derived chemokine, KC) with its receptor CXCR2 on neutrophils can induce neutrophil arrest *in vitro* and *in vivo*. [8,9]

CXCR2 is a $G\alpha_i$-coupled receptor. [10] The activation of this receptor leads to the dissociation of the α_i subunit from the β and γ subunits, and subsequent intracellular downstream signaling events. The $G\alpha_i$ family consists of the subunits $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$, which can be blocked by pertussis toxin, and $G\alpha_z$, which cannot. [11] $G\alpha_{i2}$ and $G\alpha_{i3}$ are abundantly expressed in leukocytes, and $G\alpha_{i1}$ is expressed at low levels. [12] $G\alpha_i$-mediated signaling is involved in neutrophil activation, adhesion, and recruitment. [9,13-15] Following activation of the G-protein coupled receptor (GPCR), the dissociated $G\beta\gamma$-complex can activate phosphoinositide-3 kinase (PI(3)K)γ and phospholipase C (PLC) β_2 and, to a lesser extent PLC-β_3. [16-18] PLC β hydrolyzes phosphatidylinositol 4,5-biphosphate to produce inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes Ca^{2+} from non-mitochondrial stores, and DAG activates Ca^{2+}-dependent and Ca^{2+}-independent protein kinase C (PKC) isoenzymes. A recent *in vitro* study [19] showed that activation of $\alpha_4\beta_1$ integrin on U937 monocyte-like cells and subsequent arrest is critically dependent on the activation of PLC, IP_3 receptors, increased intracellular calcium, influx of

extracellular calcium, and calmodulin. Another Gβγ-complex target, PI(3)K)γ, is not involved in neutrophil arrest *in vivo*, but is instead necessary for post-adhesion strengthening. [20] The $G\alpha_i$-subunits involved in neutrophil arrest following activation of $G\alpha_i$-coupled receptors remain unknown.

Neutrophil recruitment into the lung is different from recruitment to other tissues in that neutrophils accumulate in the lung microcirculation partially independent of adhesion molecules [21] and may not require rolling and arrest. Nevertheless, CXCR2 is critically involved in neutrophil recruitment in different models of lung inflammation. [22,23] CXCR2 on hematopoietic and non-hematopoietic cells each contributes approximately 50% to neutrophil recruitment into the lung following LPS inhalation, and no neutrophil recruitment was observed when CXCR2 is absent in all cells. [23] A recent study showed that *Gnai2*$^{-/-}$ mice reconstituted with *Gnai2*$^{+/+}$ bone marrow had defective eosinophil recruitment in a model of allergic airway inflammation, suggesting that $G\alpha_{i2}$ signaling in lung cells is necessary for eosinophil recruitment. [13] The same study also reported that elimination of $G\alpha_{i2}$ by gene targeting leads to reduced neutrophil recruitment into the lung following LPS exposure. Neutrophils from *Gnai2* deficient mice show an increased migration *in vitro* toward a chemoattractant, but the role of $G\alpha_{i2}$ in hematopoietic or non-hematopoietic cells in response to LPS was not determined. [13]

In order to test whether $G\alpha_{i2}$ is involved in chemokine-induced arrest, autoperfused flow chambers with defined substrates and intravital microscopy of cremaster venules were used. To test the physiological importance of $G\alpha_{i2}$, we investigated neutrophil recruitment in models of lipopolysaccaride (LPS)-induced pulmonary inflammation and thioglycollate-induced peritonitis. In order to distinguish between hematopoietic and non-hematopoietic $G\alpha_{i2}$, bone marrow chimeric mice were used.

Materials and Methods

Animals and generation of bone marrow chimeras

We used 8-12 weeks old *Gnai2* deficient mice and littermate control mice on the 129/Sv background. [24] Genotyping was performed using polymerase chain reaction (PCR). The wild type *Gnai2* allele was detected as a 119 bp fragment using primers Ai2E6F1 (5'-GATGTTTGATGTGGGTGGTCAGC) and Ai2Ex6B (5'-TCCTCAGCCAGCACCAAGTCATAA), while the targeted allele was detected using Ai2Ex6B and PGK-2 (5'-ACTTCCTGACTAGGGGAGGAGTAGAAGGTG), yielding a 252 bp fragment. Mice were housed in a barrier facility under specific pathogen-free conditions. The Animal Care and Use Committee of the University of Virginia (Charlottesville) approved all animal experiments. Bone marrow transplantation was performed as described previously.[25] Briefly, recipient mice were lethally irradiated in two doses of 600 rad each (separated by 4 hours). Bone marrow was isolated from donor mice under sterile conditions, and approximately 5×10^6 unfractionated cells were injected intravenously into recipient mice. Experiments were performed 6 weeks after BMT.

Surgical Preparation and intravital microscopy

Mice were anesthetized with an i.p. injection of ketamine hydrochloride (125 mg/kg, Sanofi Winthrop Pharmaceuticals, USA), atropine sulfate (0.025 mg/kg, Fujisawa, USA) and xylazine (12.5 mg/kg, Tranqui Ved, Phonix Scientific, USA) and placed on a heating pad. After tracheal intubation and cannulation of one carotid artery, the cremaster muscle was prepared for intravital microscopy as previously described. [26] Microscopic observations were made on postapillary venules (diameter 20–40 µm) using an intravital microscope (Axioskop; Zeiss, Thornwood, NY) with a saline immersion objective (SW 40/0.75 numerical aperture). A CCD camera (model VE-

1000CD, Dage-MTI) was used for recording. Leukocyte arrest was determined before and 1 minute after intravenous injection of 600ng CXCL1 (PeproTech, NJ, USA) or 5μg LTB_4 (Cayman Chemical, Michigan, USA) as described previously. [9] Arrest was defined as leukocyte adhesion longer than 30 seconds and expressed as cells per surface area. Surface area, S, was calculated for each vessel using $S=\pi*d*l_v$, where d is the diameter and l_v is the length of the vessel. Blood flow centerline velocity was measured using a dual photodiode sensor and a digital on-line cross-correlation program (Circusoft Instrumentation, Hockessin, DE). Centerline velocities were converted to mean blood flow velocities by multiplying with an empirical factor of 0.625. [27] Wall shear rates (γ_w) were estimated as 4.9 (8 vb/d), where vb is the mean blood flow velocity, d is the diameter of the vessel, and 4.9 is a median empirical correction factor obtained from velocity profiles measured in microvessels in vivo. [28]

Blood-Perfused Microflow Chamber

An autoperfused flow chamber system was used to investigate neutrophil arrest as previously described. [20,29] Rectangular glass capillaries (20 x 200 μm) were coated with P-selectin (20 μg/ml, R&D Systems, MN, USA), ICAM-1 (15 μg/ml, R&D Systems, MN, USA), and CXCL1 (15 μg/ml, PeproTech, NJ, USA) for 2 h and blocked for 1h using 10% casein (Pierce Chemicals, Dallas, TX). Each capillary was connected to a PE 10 catheter (Becton Dickinson, Sparks, MD) inserted into the carotid artery. The other side of the chamber was connected to a PE 50 tubing and used to control the wall shear stress, which was calculated as described. [29] Each chamber was perfused with blood for 6 min before one representative field of view was recorded for 1 min. using an SW40/0.75 objective. In some experiments, blood was collected by cardiac puncture and phospholipase C was blocked with the pharmacological inhibitor U73122 (1μM,

Cayman Chemical, Ann Arbor, MI) for 30 minutes. U-73122 is an inhibitor of PLC-dependent processes, however, the mechanism of action remains unclear. [30-32]

Quantitative real-time RT-PCR

Total RNA from isolated neutrophils and lymphocytes was extracted using Trizol (Invitrogen, Carlsbad, CA). Reverse transcription was performed using an Omniscript kit (Qiagen, Valencia, CA), oligo DT primers and 150 ng of total RNA. Real-time PCR was performed on an iCycler iQ Real-time Detection System (Qiagen, Valencia, CA) with sequence specific primers and Taqman® probes designed on Beacon Designer 7 software (Biosoft International, Palo Alto, CA). One microliter of cDNA was used for all samples, which were run in triplicate. Values were determined using iCycler iQ Real-time Detection System Software v3.1 (Qiagen, Valencia, CA). The resulting values were normalized to GAPDH.

Murine Model of LPS-induced pulmonary inflammation

As described previously, [33] LPS from *Salmonella enteritidis* (500 µg/ml, Sigma-Aldrich) was nebulized to induce pulmonary inflammation. Briefly, mice were exposed 30 minutes to aerosolized LPS or saline aerosol as a control. 24h after inhalation, mice were euthanized and neutrophil recruitment into the alveolar compartment was determined by bronchoalveolar lavage (BAL). BAL fluid was recovered after instillation of phosphate-buffered saline (5 x 1ml). BAL was centrifuged and leukocytes were counted using Kimura stain. The fraction of neutrophils in the suspension was determined by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA). Neutrophils were identified by their typical appearance in the forward / sideward scatter (FSC/SSC) and their expression of CD45 (clone 30-F11), 7/4 (clone 7/4, both BD Biosciences-Pharmingen, San Diego, CA), and GR-1 (clone RB6-8C5).

Peritonitis Model

Peritoneal recruitment of leukocytes was induced using 4% thioglycollate (Sigma-Aldrich, MO, USA) according to previously published methods. [9] After 4 h, mice were killed, the peritoneal cavity was rinsed with 10 ml PBS (containing 2 mM EDTA), and fluid was analyzed for the number of neutrophils using Kimura-staining.

Statistics

Statistical analysis was performed with SPSS (version 14.0, Chicago, IL) and included one-way analysis of variance, Student-Newman-Keuls test, and t-test where appropriate. All data are presented as mean ± SEM. $P < 0.05$ were considered significant.

Results

Gα_{i2} and phospholipase C in neutrophils are required for neutrophil chemokine-induced arrest on P-selectin/ICAM and calcium influx.

Whole native mouse blood was perfused over P-selectin and ICAM-1 co-immobilized with CXCL1 at a wall shear stress of 5.94 dyn/cm^2. In a previous study, we showed that over 90% of the rolling cells in this model are neutrophils. [29] A few neutrophils from *Gnai2*$^{+/+}$ mice, but none from *Gnai2* deficient mice adhered to P-selectin and ICAM-1 coated flow chambers (Figure 1A). In *Gnai2*$^{+/+}$ mice, but not in *Gnai2*$^{-/-}$ mice, neutrophil adhesion significantly increased when CXCL1 was co-immobilized with P-selectin and ICAM-1 (Figure 1A).

Phospholipase C can be activated by the Gβγ-complex following activation of GPCRs and is involved in the regulation of the integrin affinity state and arrest of monocyte-like U937 cells. [19] In order to address whether PLC is also involved in chemokine-induced arrest of primary

mouse neutrophils, neutrophil arrest was investigated in the flow chamber system following blocking of PLC by incubating whole blood with a pharmacological inhibitor. Incubation of blood from *Gnai2*$^{+/+}$ mice with the PLC inhibitor U73122 completely eliminated neutrophil arrest in flow chambers coated with P-selectin, ICAM-1, and CXCL1 (Figure 1B). Similar to the ex vivo perfused findings (Figure 1A), blood from *Gnai2* deficient mice treated with the PLC inhibitor showed no adherent neutrophils (Figure 1B).

Reduced chemokine-induced arrest in cremaster venules of Gnai2 deficient mice in vivo.

In order to confirm our *in vitro* data *in vivo*, we conducted intravital microscopy of the cremaster muscle. In the acutely exteriorized mouse cremaster, neutrophils roll along the endothelium of venules, but neutrophil adhesion is almost absent. [26] Neutrophil rolling in this model is known to be mediated by P-selectin. [26] Injection of the recombinant murine chemokine CXCL1, which binds and activates CXCR2, induced immediate firm arrest in *Gnai2*$^{+/+}$ mice (Figure 2A). *Gnai2*$^{-/-}$ mice showed the same number of adherent neutrophils under baseline conditions as *Gnai2*$^{+/+}$ mice when accounting for their elevated blood neutrophil numbers. In contrast to *Gnai2*$^{+/+}$ mice, the number of adherent cells per area in *Gnai2*$^{-/-}$ mice did not increase after CXCL1 injection (Figure 2A). Representative video micrographs of *Gnai2*$^{+/+}$ mice and *Gnai2*$^{-/-}$ mice before and after CXCL1 treatment are shown in Figure 2B. Wall shear rates and diameters were similar in the investigated venules, excluding a hemodynamic contribution to reduced neutrophil adhesion (data not shown).

Previous studies have shown that some interventions like blocking phosphoinositide-3 kinase (PI(3)k) γ, [20] Vav1 and 3, [34] or Src kinases [35] impair post-adhesion strengthening, which leads to neutrophil detachment after adhesion. To test whether neutrophils from *Gnai2*$^{-/-}$ mice have an arrest defect or a post-adhesion strengthening defect, adherent neutrophils were tracked

after CXCL1 injection. In *Gnai2*$^{+/+}$ mice, neutrophils adhered rapidly to the endothelium following CXCL1 injection and remained attached over the next minute (Figure 2C). *Gnai2*$^{-/-}$ mice showed also rapid arrest after CXCL1 injection, but at much lower numbers (Figure 2D). Adhesion stability was unaffected. These data demonstrate that $G\alpha_{i2}$ signaling is required for neutrophil arrest but is probably not involved in adhesion strengthening.

Gnai2 deficient mice cannot induce leukocyte arrest in postcapillary venules of the cremaster muscle in response to LTB_4.

In order to extend our findings to a different chemoattractant receptor, we used leukotriene B_4 (LTB_4), which binds to and activates BLTR1, a $G\alpha_i$-coupled receptor. [36] LTB_4 induced leukocyte arrest in *Gnai2*$^{+/+}$ mice (Figure 3). However, in *Gnai2*$^{-/-}$ mice, LTB_4 induced very little leukocyte adhesion. To test possible compensatory mechanisms in these mice, we measured mRNA for $G\alpha_{i3}$ by real time RT-PCR and found no evidence for up-regulation in *Gnai2*$^{-/-}$ mice (data not shown).

$G\alpha_{i2}$ in neutrophils is responsible for chemokine-induced neutrophil arrest.

The autoperfused flow chamber data suggested that neutrophil $G\alpha_{i2}$ is required for CXCL1-induced arrest. To directly test the role of neutrophil $G\alpha_{i2}$ in chemokine-induced arrest *in vivo*, bone marrow from *Gnai2* deficient mice was transplanted into lethally irradiated *Gnai2*$^{+/+}$ mice. In these mice, bone marrow-derived cells lack $G\alpha_{i2}$, but endothelial and other cells express $G\alpha_{i2}$. Leukocyte arrest in response to intravenous CXCL1 was investigated by intravital microscopy in postcapillary cremaster venules 6 to 8 weeks after transplantation. CXCL1 induced leukocyte adhesion in *Gnai2*$^{+/+}$ control mice. In *Gnai2*$^{-/-}$ → *Gnai2*$^{+/+}$ chimeric mice, CXCL1-induced neutrophil arrest was significantly reduced but not abolished (Figure 4).

Neutrophil recruitment in a model of LPS-induced lung inflammation and thioglycollate-induced peritonitis is partially dependent on $G\alpha_{i2}$ in neutrophils.

Previous studies have shown that blocking of $G\alpha_i$ by PTx reduced neutrophil recruitment into inflamed tissue. [9,15] However, PTx treatment blocks hematopoietic as well as non-hematopoietic $G\alpha_i$. In view of recent findings of an important role for $G\alpha_{i2}$ [13] and CXCR2 [23] in non-hematopoietic cells, we wished to differentiate between the role of hematopoietic and non-hematopoietic $G\alpha_{i2}$ for neutrophil recruitment. We used bone marrow chimeric mice to investigate neutrophil recruitment. In a model of LPS-induced lung inflammation, *Gnai2*$^{+/+}$ mice showed a significant increase of neutrophil recruitment into the alveolar compartment 24h after LPS inhalation (Figure 5A). Neutrophil influx was reduced by approximately 50% in *Gnai2*$^{+/+}$ mice that were lethally irradiated and reconstituted with *Gnai2*$^{-/-}$ bone marrow (Figure 5A). Control mice receiving *Gnai2*$^{+/+}$ bone marrow showed no recruitment defect. To test the physiologic importance of $G\alpha_{i2}$ in bone marrow derived cells in a second model, we investigated thioglycollate-induced peritonitis. Neutrophil influx into thioglycollate-induced peritonitis was significantly reduced in *Gnai2*$^{-/-}$ compared to *Gnai2*$^{+/+}$ (Figure 5B). Bone marrow chimeric mice that had received *Gnai2*$^{-/-}$ bone marrow showed a similar decrease of neutrophil recruitment into the peritoneal cavity as *Gnai2*$^{-/-}$ mice (Figure 5B). These data show that neutrophil $G\alpha_{i2}$ is relevant *in vivo*.

Discussion

Many studies have shown that GPCRs are involved in triggering neutrophil arrest under flow, [9,20,37-39] but the specific Gα subunit used was not known. Here, we demonstrate that $G\alpha_{i2}$ in neutrophils is required for chemokine-induced arrest under flow in response to CXCL1. The physiologic importance of $G\alpha_{i2}$ in bone marrow-derived cells is demonstrated by significant

defects in neutrophil recruitment in an LPS-induced model of lung inflammation and in thioglycollate-induced peritonitis.

In a recent study, Pero et al. showed that eosinophil recruitment in a model of allergic airway inflammation was almost abolished in mice lacking $G\alpha_{i2}$. [13] The trafficking defect could not be restored when *Gnai2*$^{-/-}$ mice were reconstituted with *Gnai2*$^{+/+}$ bone marrow, suggesting that endothelial cell $G\alpha_{i2}$ was required for transmigration. In the same study, [13] a neutrophil recruitment defect was seen in *Gnai2*$^{-/-}$ mice in an LPS-induced model of lung inflammation, but the role of $G\alpha_{i2}$ on bone marrow-derived cells and other cells was not addressed separately. In a study of CXCR2$^{-/-}$ mice, CXCR2 was found to be required on both neutrophils and endothelial cells in order to support optimal neutrophil recruitment to the bronchoalveolar space in response to aerosolized LPS. [23] The present results demonstrate that $G\alpha_{i2}$ is also required on both bone marrow-derived and other cells for neutrophil recruitment in LPS-induced lung inflammation. The reason for the greater importance of endothelial cell $G\alpha_{i2}$ and lesser importance of eosinophil $G\alpha_{i2}$ in eosinophil recruitment in allergic airway inflammation [13] remains to be explored.

$G\alpha_i$-coupled GPCRs are involved in many biological functions of neutrophils, including adhesion, [9] triggering the respiratory burst, [40] degranulation [41] and actin polymerization. [42] The focus of the present study was on neutrophil arrest, one of the earliest steps in the inflammatory adhesion cascade. [1,2] While our findings of reduced neutrophil recruitment into the inflamed lung and peritoneal cavity of mice reconstituted with *Gnai2*$^{-/-}$ bone marrow are consistent with the observed arrest defect, it is possible that defective chemotaxis and transendothelial migration of *Gnai2*$^{-/-}$ neutrophils also contribute to the observed phenotype. Since neutrophil availability in the blood, arrest and chemotactic migration are considered sequential events, the disturbance of one of these factors can modulate the recruitment of neutrophil into the tissue. In the case of LPS-induced lung inflammation, this is indeed likely, because CXCR2 is not required for neutrophil

accumulation in the lung vasculature. [23] The lung microcirculation has very small capillaries, allowing neutrophils to lodge even without adhesion mechanisms. [21] $G\alpha_{i2}$ is also critically involved in chemokine receptor signaling in lymphocytes. [43,44] B- [44] and T-lymphocytes [43] from *Gnai2* deficient mice show reduced chemotaxis and homing to lymph nodes. The more severe increase of lymphocyte than neutrophil numbers in *Gnai2* deficient mice suggests that lymphocytes rely more on chemokine signals than neutrophils.

Our study establishes $G\alpha_{i2}$ as the most important Gα subunit in chemoattractant-induced neutrophil arrest. This is consistent with previous work [9] showing that neutrophil arrest is blocked by pertussis toxin, an intervention that blocks all $G\alpha_i$ signaling. However, it remains unclear whether $G\alpha_{i2}$ directly triggers downstream signaling events, or whether it releases a specific subset of Gβγ-subunits that are uniquely suited to activate other signaling molecules. A recent study showed that PLC is required for rapid arrest in response to chemokine stimulation of monocyte-like U937 cells. [19] Our study extends these findings to neutrophils and strongly suggests that Gβγ-subunits released by $G\alpha_{i2}$, but not $G\alpha_{i3}$ are able to activate PLC. The specific PLC isoform involved and the signaling steps downstream from PLC that ultimately link GPCRs to integrin activation remain to be determined.

Gnai2$^{-/-}$ mice clearly have residual neutrophil function, but blood neutrophil numbers are elevated in these mice. Elevated neutrophil counts are a typical compensatory response to neutrophil recruitment defects. [45,46] Indeed, microvessels in *Gnai2*$^{-/-}$ mice contain more adherent neutrophils than those of normal mice. [13] This suggests that alternative, GPCR-independent adhesion mechanisms are important for neutrophil recruitment. One such mechanism is triggered by E-selectin binding to PSGL-1, which leads to LFA-1 activation through a pathway that requires spleen tyrosine kinase. [5] Another GPCR-independent pathway for neutrophil activation during rolling requires ESL-1 and CD44. [47] It is likely that these GPCR-independent mechanisms

account for at least some of the residual neutrophil recruitment seen in *Gnai2*$^{-/-}$ mice. Interestingly, $G\alpha_{i2}$ does not appear to be involved in post-arrest adhesion strengthening. This step involves integrin rearrangement in the plasma membrane, clustering and outside-in signaling. [48,49] The few neutrophils that arrest in postcapillary venules of *Gnai2*$^{-/-}$ mice are just as firmly adhered as wild-type neutrophils, suggesting that the GPCR-independent neutrophil activation pathways are fully capable of producing stable adhesion *in vivo*. This is in contrast to observations in reconstituted flow chamber systems, where E-selectin engagement of PSGL-1 can only trigger partial activation of LFA-1, which induces slow rolling, but not arrest. [5] These data suggest that some unknown, additional neutrophil activating signal(s) seem to be present *in vivo*. The present data show that these additional signals function independent of $G\alpha_{i2}$.

Leukocyte arrest under flow is usually triggered by surface-immobilized chemokines, [50,51] but soluble chemoattractants are equally effective. For example, fMLP injected intravenously induces immediate arrest. [39] Here, we show that at least one soluble chemoattractant, LTB_4, also uses $G\alpha_{i2}$ to trigger arrest. Since the inhibition of arrest was not complete, it is possible that BLTR1, the receptor for LTB_4, coupled to another G-protein different from $G\alpha_{i2}$ that may be responsible for the residual effect.

Our study establishes $G\alpha_{i2}$ as the main G-protein α subunit required for chemoattractant-induced neutrophil arrest in vitro and in vivo. Further studies will be required to fully unravel the signaling pathway that links GPCRs to integrin activation in neutrophils.

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Author's contributions

Alexander Zarbock: designed experiments, performed research and wrote the paper.

Tracy L. Deem: performed flow chamber experiments.

Tracy L. Burcin: performed RT-PCR.

Klaus Ley: designed experiments and wrote parts of the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Legends

Figure 1: **Gα_{i2} and phospholipase C in neutrophils are required for neutrophil chemokine-induced arrest on P-selectin/ICAM.** (A) Carotid cannulas were placed in *Gnai2*$^{-/-}$ mice (open bars) and *Gnai2*$^{+/+}$ mice (filled bars) and connected to autoperfused flow chambers which coated with P-selectin and ICAM-1 alone or in combination with CXCL1 (10 µg/ml). The wall shear stress in all flow chamber experiments was 5.94 dynes/cm^2. At least three mice and four flow chambers per group. To account for the 2.5 fold increase of neutrophils in *Gnai2* deficient mice compared to littermate controls (Table 1A), the number of adherent neutrophils was normalized to systemic neutrophil counts. Number of normalized adherent cells per field of view is presented as mean ± SEM. (B) Number of adherent neutrophils on P-selectin and ICAM-1 in combination with CXCL1 of U73122-pretreated whole blood after 6 minutes. Data presented are the mean ± SEM from 3 mice. $^{\#}$ $P < 0.05$.

Figure 2: **Reduced chemokine-induced arrest in cremaster venules of *Gnai2* deficient mice *in vivo*.** (A) Number of adherent cells (normalized to blood neutrophil counts) in cremaster muscle postcapillary venules of *Gnai2*$^{-/-}$ mice and littermate control mice after i.v. injection of 600 ng CXCL1. Data were recorded and analyzed for 1 min starting 15 sec. after CXCL1 injection. Data presented are the mean ± SEM from four mice. (B) Representative pictures of cremaster muscle postcapillary venules of *Gnai2*$^{+/+}$ and *Gnai2*$^{-/-}$ mice before and 1 min after CXCL1 injection (leukocytes are circuited: rolling leukocytes: dotted line, arrested leukoctes: solid line). Scale bar equals 10 µm. (C and D) Adherent leukocytes in postcapillary venules of *Gnai2*$^{-/-}$ mice (D) or littermate control mice (C) after injection of CXCL1. Each line represents the number of adherent leukocytes per mm^2 in one venule of one mouse after CXCL1 injection. Data presented are the mean ± SEM from four mice. $^{\#}$ $P < 0.05$.

Figure 3: ***Gnai2* deficient mice cannot induce leukocyte arrest in postcapillary venules of the cremaster muscle in response to LTB_4.** Number of adherent leukocytes in cremaster muscle postcapillary venules of *Gnai2*$^{-/-}$ mice and *Gnai2*$^{+/+}$ mice after i.v. injection of 5µg LTB_4. Data were recorded and analyzed for 1 min starting 15 sec. after CXCL1 injection._Data presented are the mean ± SEM from four mice. $^{\#}P < 0.05$.

Figure 4: **$G\alpha_{i2}$ in neutrophils is responsible for chemokine-induced neutrophil arrest.** Number of adherent leukoctes in cremaster muscle postcapillary venules of *Gnai2*$^{-/-}$ → *Gnai2*$^{+/+}$ chimeric mice and *Gnai2*$^{+/+}$ mice after i.v. injection of 600 ng CXCL1 (normalized for blood neutrophil counts, A). Data were recorded and analyzed for 1 min starting 15 sec. after CXCL1 injection. Data presented as mean ± SEM from at least four mice.

Figure 5: **Neutrophil recruitment in a model of LPS-induced lung inflammation and thioglycollate-induced peritonitis is partially dependent on $G\alpha_{i2}$ in neutrophils.** (A) Neutrophil recruitment into the alveolar compartments of the lung with or without LPS inhalation (24h) was determined by flow cytometry. *Gnai2*$^{-/-}$ mice, *Gnai2*$^{+/+}$, and *Gnai2*$^{-/-}$ → *Gnai2*$^{+/+}$ chimeric mice showed different recruitment patterns of neutrophils in the alveolar compartment (n=4). (B) Peritoneal neutrophil influx 4 h after injection of 4% thioglycollate into *Gnai2*$^{-/-}$ (four mice), *Gnai2*$^{-/-}$ (three mice), *Gnai2*$^{-/-}$ → *Gnai2*$^{+/+}$ chimeric mice (five mice), and *Gnai2*$^{+/+}$ → *Gnai2*$^{+/+}$ (five mice). Total number of neutrophils ($x10^6$) in the peritoneal cavity counted using Kimura-stained samples. $^{*}P < 0.05$ versus other groups, $^{\#}P < 0.05$.

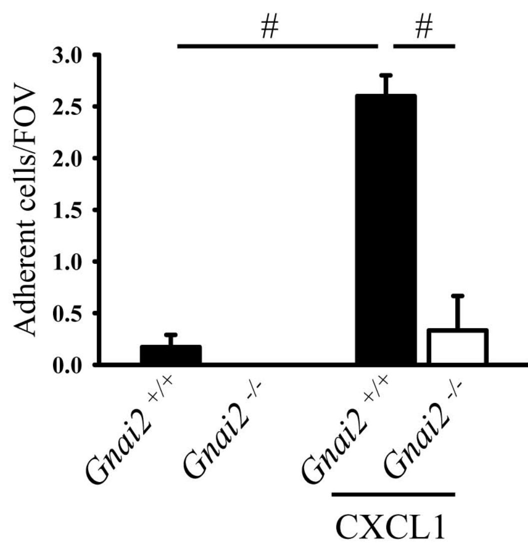
Table 1: **Systemic leukocyte blood counts.** Leukocyte, neutrophil, lymphocyte, and monocte counts of at least 5 mice per group. Data are presented as mean ± SEM. *P < 0.05 versus *Gnai2*$^{+/+}$.

Table 1

	Leukocytes (x 10^3 cells/µl)	Neutrophils (x 10^3 cells/µl)	Lymphocytes (x 10^3 cells/µl)	Monocytes (x 10^3 cells/µl)
Gnai2$^{+/+}$	6.1 ± 2.2	1.0 ± 0.8	4.6 ± 1.2	0.6 ± 0.2
Gnai2$^{-/-}$	24.2 ± 5.3 *	2.5 ± 1.7	18.8 ± 3.2 *	2.8 ± 2.0
Gnai2$^{+/+}$ → *Gnai2*$^{+/+}$	7.2 ± 1.0	1.1 ± 0.2	5.4 ± 0.6	0.6 ± 0.4
Gnai2$^{-/-}$ → *Gnai2*$^{+/+}$	13.2 ± 2.5	1.4 ± 0.2	10.7 ± 2.2 *	1.2 ± 0.3

Figure 1

A



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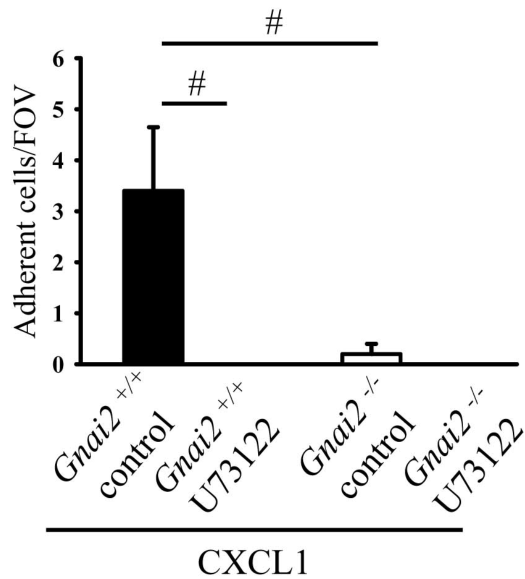

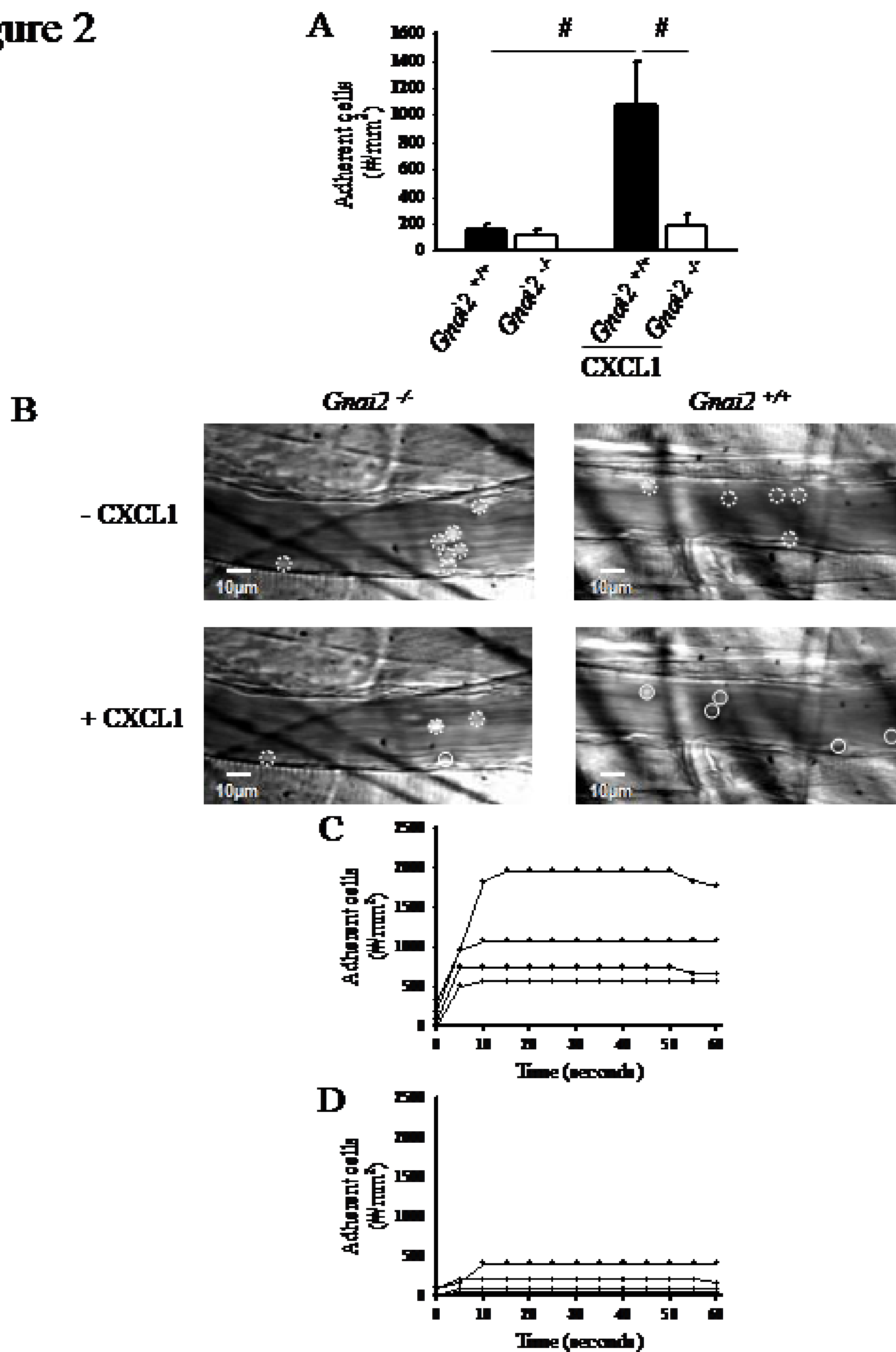

Figure 2
A
Adherent cells (#/mm²)
1600
1400
1200
1000
800
600
400
200
0
#
#
Gnai2 +/+
Gnai2 -/-
Gnai2 +/+
Gnai2 -/-
CXCL1
B
Gnai2 -/-
Gnai2 +/+
- CXCL1
+ CXCL1
10µm
10µm
10µm
10µm
C
Adherent cells (#/mm²)
Time (seconds)
D
Adherent cells (#/mm²)
Time (seconds)

Figure 3

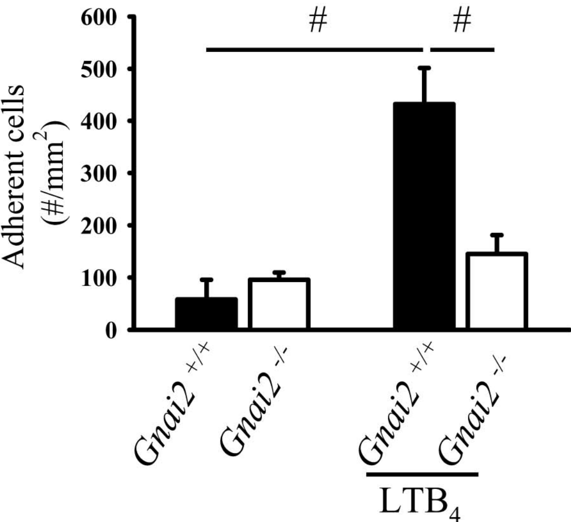

Figure 4

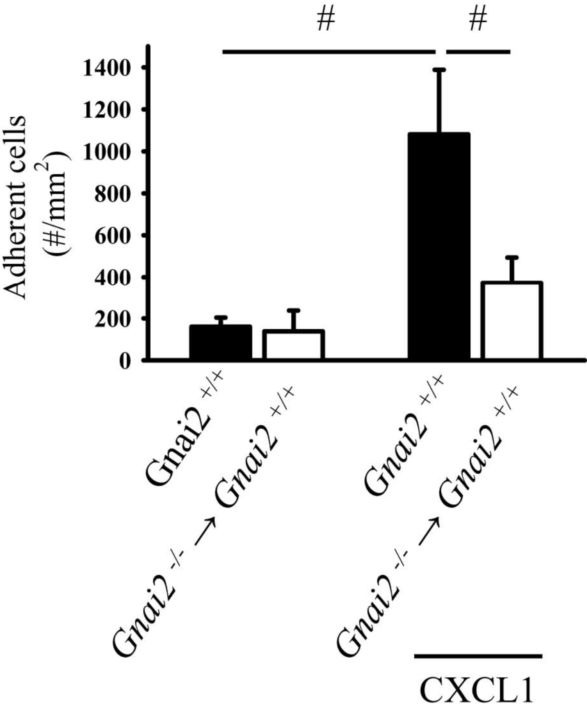

Figure 5