

Critical role of phospholipase C γ 2 in integrin and Fc receptor-mediated neutrophil functions and the effector phase of autoimmune arthritis

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β_2 integrins and Fc γ receptors are critically involved in neutrophil activation at the site of inflammation. Both receptor types trigger a receptor-proximal tyrosine phosphorylation cascade through Src family kinases and Syk, but further downstream signaling events are poorly understood. We show that phospholipase C (PLC) γ 2 is phosphorylated downstream of Src family kinases and Syk during integrin or Fc receptor-mediated activation of neutrophils. PLC γ 2^{-/-} neutrophils are completely defective in β_2 integrin or Fc γ receptor-mediated functional responses such as respiratory burst, degranulation, or cell spreading in vitro and show reduced adhesion/spreading in inflamed capillary venules in vivo. However, PLC γ 2^{-/-} neutrophils respond normally to various other agonists, including chemokines, bacterial formyl peptides, Toll-like receptor ligands, or proinflammatory cytokines, and migrate normally both in vitro and in vivo. To confirm the in vivo relevance of these observations, the effect of the PLC γ 2^{-/-} mutation was tested in the K/B \times N serum transfer arthritis model, which is known to require β_2 integrins, Fc γ receptors, and neutrophils. PLC γ 2 deficiency completely protected mice from clinical signs and histological features of arthritis as well as from arthritis-induced loss of articular function. These results identify PLC γ 2 as a critical player of integrin and Fc receptor-mediated neutrophil functions and the neutrophil-mediated effector phase of autoimmune arthritis.

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Abbreviations used: ERK, extracellular signal-regulated kinase; ITAM, immunoreceptor tyrosine-based activation motif; PLC, phospholipase C.

Neutrophils play a critical role in innate immune defense, but their improper activation also contributes to tissue damage during autoimmune diseases such as rheumatoid arthritis (1–5). Neutrophils use several cell surface receptors to sense their environment including β_2 integrins, immunoglobulin Fc receptors, various G protein-coupled (e.g., formyl peptide or chemokine) receptors, Toll-like receptors, and receptors for various proinflammatory cytokines.

Lymphocyte antigen receptors, Fc ϵ receptors of mast cells, and Fc γ receptors of macrophages use a common receptor-proximal signal transduction machinery consisting of the sequential activation of Src family kinases, immunoreceptor tyrosine-based activation motif (ITAM) containing transmembrane adapters, and the Syk or the ZAP-70 tyrosine kinase. Studies from other groups (6, 7) and our own unpublished

observations indicate that neutrophil Fc γ receptors also use a receptor-proximal Src family-ITAM-bearing adaptor-Syk signaling pathway. We have recently shown that β_2 integrins in neutrophils signal through a conceptually similar receptor-proximal pathway, using Src family kinases (8, 9), two ITAM-bearing transmembrane adapters (DAP12 and the Fc receptor γ chain) (10), and the Syk tyrosine kinase (11). We and others have reported similar ITAM-based integrin signaling pathways in other cell types including macrophages (10), platelets (12), osteoclasts (13), dendritic cells (14), and microglia (15). Collectively, integrins and Fc receptors in

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various hematopoietic lineages signal through a conceptually similar ITAM-based receptor-proximal tyrosine phosphorylation cascade (for review see reference 16). However, the signal transduction mechanisms downstream of this common receptor-proximal pathway are poorly understood.

Phosphoinositide-specific phospholipase C (PLC) enzymes catalyze the breakdown of the membrane lipid phosphatidylinositol-4,5-bisphosphate to inositol-3,4,5-trisphosphate and diacylglycerol, triggering a concomitant Ca^{2+} signal and protein kinase C activation. Of the best known PLC isoforms, the PLC β family is activated by G protein-coupled receptors, whereas the PLC γ family is activated downstream of tyrosine phosphorylation pathways. There are two known PLC γ isoforms: PLC γ 1 is ubiquitously expressed, whereas PLC γ 2 is preferentially expressed in the hematopoietic system. Genetic deficiency of PLC γ 1 leads to embryonic lethality, likely as a result of defective erythropoiesis and vasculogenesis (17, 18). In contrast, PLC γ 2-deficient mice are viable, their principal phenotype being a profound defect in B cell development and function (19).

Although PLC γ 2 is activated by various Fc receptors, its possible functional role downstream of those receptors is rather controversial. Genetic deficiency of PLC γ 2 attenuates Fc ϵ receptor-mediated degranulation of mast cells (19, 20) but it does not affect extracellular signal-regulated kinase (ERK) activation or cytokine production under the same conditions (20). Although PLC γ 2 is required for Fc γ receptor-triggered Ca^{2+} signal in macrophages, PLC γ 2 $^{-/-}$ macrophages show normal phagocytosis of IgG-coated erythrocytes (20). The role of PLC γ 2 in Fc receptor-mediated functions in other cell types such as neutrophils is presently unknown.

PLC γ 2 is also activated by integrins but its role in integrin signal transduction is also controversial. Although a statistically significant decrease of spreading was reported in PLC γ 2 $^{-/-}$ platelets (21, 22), that difference only accounted for a 30% reduction of the $\alpha_2\beta_1$ integrin-induced increase in cell surface area (21) or a delayed kinetics and moderately smaller percentage of full spreading on an $\alpha_{IIb}\beta_3$ integrin ligand surface (22). Hence, PLC γ 2 appears to be a modulator rather than a critical component of integrin signaling in platelets. In contrast, a recent study focusing on the role of Vav family proteins in neutrophils suggested that PLC γ 2 downstream of Vav may be more directly involved in integrin signaling in these cells (23).

Rheumatoid arthritis is a severe chronic autoimmune disease affecting $\sim 1\%$ of the human population (24). The disease is initiated by the emergence of autoreactive T cells (initiation or immunization phase), which then trigger the second (effector or tissue destruction) phase, mediated in large part by cells of the innate immune system. These two phases are very clearly separated in the K/B \times N arthritis model (25). This model is initiated by a transgenic autoreactive T cell receptor (KRN transgene) on the autoimmunity-prone MHC background from the NOD mouse strain. This initial phase leads to the generation of autoantibodies that trigger excessive joint inflammation and destruction resembling human

rheumatoid arthritis. Serum of affected mice can trigger the effector phase of the disease in otherwise normal mice (serum transfer arthritis) (26), allowing a clear separation of the two phases of the disease.

Analysis of the K/B \times N and other models of autoimmune arthritis revealed that innate immune mechanisms are of critical importance in the later effector phase of the disease (26). Several studies using lineage depletion (27–29), genetic (30, 31), or combined genetic/reconstitution (32, 33) approaches indicate that neutrophils play a critical role in the effector phase of various animal models of autoimmune arthritis. However, the molecular mechanisms of how neutrophils contribute to the disease are very poorly understood.

Several cell surface receptors have been shown to be involved in the pathogenesis of autoimmune arthritis in mice. These receptors include Fc γ receptors such as Fc γ RIII or Fc γ RI (34–43) as well as members of the β_2 integrin family (44, 45). However, it is at present unclear how (e.g., through what intracellular signaling mechanisms) Fc receptors and integrins participate in the development of joint inflammation.

The aforementioned results prompted us to test the role of PLC γ 2 in various *in vitro* neutrophil functions as well as in the development of neutrophil-mediated autoimmune arthritis *in vivo*. Our results indicate that PLC γ 2 is critically involved in integrin and Fc receptor-mediated neutrophil functions as well as in the neutrophil-mediated effector phase of autoimmune arthritis.

RESULTS

PLC γ 2 is the dominant PLC γ isoform and is phosphorylated downstream of Src family kinases and Syk in neutrophils

First, we tested the expression level of the two PLC γ isoforms in neutrophils and compared it to that in other cell types. As shown in Fig. 1 A, PLC γ 2 was expressed at comparable levels in WT murine neutrophils and splenocytes but at much lower levels in WT thymocytes. In contrast, PLC γ 1 was expressed in neutrophils at a much lower level than in the thymus or the spleen. Although these results suggested that PLC γ 2 is the predominant PLC γ isoform in neutrophils, they did not allow the quantitative assessment of the relative expression of the two proteins in these cells. Hence, the expression of PLC γ 1 and PLC γ 2 was titrated against known amounts of recombinant Myc-tagged versions of the two proteins (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20081859/DC1>). Based on those studies, WT mouse neutrophils were estimated to contain 53 ± 26 ng PLC γ 2 ($n = 3$) and 3.0 ± 1.2 ng PLC γ 1 ($n = 3$) per 10^6 cells, indicating that the expression of PLC γ 2 is ~ 18 -fold higher than that of PLC γ 1 in these cells. The expression of the two isoforms was also tested in PLC γ 2 $^{-/-}$ neutrophils (Fig. 1 B). Although no PLC γ 2 signal was observed in PLC γ 2 $^{-/-}$ cells, the expression of PLC γ 1 was not affected by the same mutation. The specificity of the antibodies used was also confirmed by the blocking effect of isoform-specific PLC γ -blocking peptides (Fig. S1 B). Collectively, these results indicate that

neutrophils express both PLC γ 1 and PLC γ 2, although PLC γ 2 is the predominant isoform. Furthermore, the genetic deficiency of PLC γ 2 does not affect the expression of PLC γ 1 in these cells.

We and others have previously shown that β_2 integrins signal through a receptor-proximal tyrosine phosphorylation cascade involving Src family kinases and Syk (8–11; for review see reference 16). Next, we tested whether PLC γ 2 is also phosphorylated under these conditions and whether Src family kinases and Syk participate in this process. As shown in Fig. 1 (C and D), plating WT neutrophils on a polyvalent

integrin ligand surface (poly-RGD) (11) triggered phosphorylation of PLC γ 2. Importantly, this phosphorylation response was absent in cells lacking the Src family kinases *Hck*, *Fgr*, and *Lyn* (Fig. 1 C) or the Syk tyrosine kinase (Fig. 1 D). Murine neutrophils can also be activated in an Fc γ RIII/Fc γ RIV-dependent manner by plating them on immobilized IgG immune complexes (46), leading to cellular responses that are dependent on Src family kinases and Syk (unpublished data). As shown in Fig. 1 (E and F), neutrophil activation by such immobilized immune complexes leads to phosphorylation of PLC γ 2 in WT but not in Src family-deficient (Fig. 1 E) or Syk-deficient (Fig. 1 F) neutrophils. These results suggest that PLC γ 2 is a downstream target of Src family kinases and Syk during both integrin and Fc receptor-mediated activation of neutrophils.

PLC γ 2^{-/-} bone marrow chimeras and neutrophil surface marker expression

Our next aim was to test the role of PLC γ 2 in functional responses of neutrophils, using cells that are genetically deficient of this phospholipase isoform. Because we (and others) have not been able to breed homozygous PLC γ 2^{-/-} mice (indicating a fertility defect in PLC γ 2^{-/-} males and/or females), the mutation was maintained in heterozygous (PLC γ 2^{+/-}) form. Even under such conditions, only 12% (rather than the expected 25%) of a total of 379 offsprings from PLC γ 2^{+/-} \times PLC γ 2^{+/-} matings were found to be of the PLC γ 2^{-/-} genotype at weaning age, indicating a partial defect in survival of PLC γ 2^{-/-} embryos or newborn pups, which is likely a result of a lymphatic vascular developmental defect (47) similar to that seen in SLP-76^{-/-} and Syk^{-/-} mice (48). To overcome this problem, bone marrow transplantation was used to generate chimeric mice with a PLC γ 2^{-/-} hematopoietic system. To this end, recipient mice carrying the CD45.1 allele on the C57BL/6 genetic background were lethally irradiated and then injected intravenously with isolated PLC γ 2^{-/-} or control C57BL/6 bone marrow cells (both donor strains carry the CD45.2 allele). Repopulation of the neutrophil compartment by donor-derived cells was confirmed by flow cytometric analysis of the donor-specific CD45.2 allele in peripheral blood leukocytes 4–5 wk after transplantation (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20081859/DC1>). Using this approach, 99.3 \pm 1.6% ($n = 120$) and 99.1 \pm 2.4% ($n = 135$) of peripheral blood neutrophils of WT and PLC γ 2^{-/-} bone marrow chimeras, respectively, were found to be of donor origin. Though quite laborious, this approach allowed us to significantly increase the number of mice available for our studies. Unless otherwise stated, all of the following experiments were performed using such WT and PLC γ 2^{-/-} bone marrow chimeras.

We next tested whether the deficiency of PLC γ 2 affected neutrophil development or expression of major cell surface receptors. Our bone marrow neutrophil isolation protocol yielded 12.1 \pm 3.3 $\times 10^6$ WT and 12.4 \pm 4.8 $\times 10^6$ PLC γ 2^{-/-} neutrophils per mouse ($n = 23$; $P = 0.62$), indicating that the lack of PLC γ 2 did not cause a quantitative change in

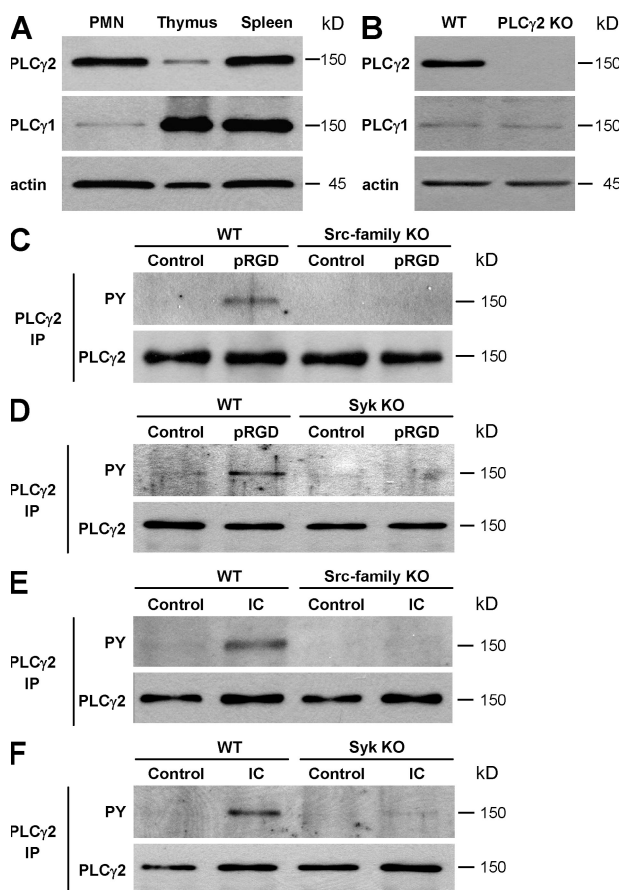


Figure 1. Expression and activation of PLC γ 2 in neutrophils.

(A) Expression of PLC γ 2 and PLC γ 1 in WT neutrophils compared with WT thymocytes and splenocytes. (B) Analysis of PLC γ 1 and PLC γ 2 expression in WT and PLC γ 2^{-/-} (PLC γ 2 KO) neutrophils. (C and D) PLC γ 2 phosphorylation in WT, Src family-deficient (*Hck*^{-/-}*Fgr*^{-/-}*Lyn*^{-/-}; Src-family KO), or Syk^{-/-} (Syk KO) neutrophils plated on a polyvalent integrin ligand (poly-RGD)-coated surface (pRGD) or left in suspension (control). PLC γ 2 phosphorylation was tested by immunoprecipitation (IP) followed by immunoblotting with antibodies against phosphotyrosine (PY). (E and F) Phosphorylation of PLC γ 2 in neutrophils of the various genotypes plated on an IgG immune complex-coated (IC) or control-treated surface. Immunoblotting for actin (A and B) and PLC γ 2 (C–F) served as loading controls. Molecular mass values represent the estimated apparent molecular mass of the proteins. Each panel represents three to five independent experiments with similar results.

neutrophil production. PLC γ 2^{-/-} neutrophils also expressed normal levels of the Gr1 granulocyte differentiation marker (Fig. 2 A) and the general leukocyte marker CD45 (Fig. S2). The PLC γ 2^{-/-} mutation did not affect expression of the β ₂ integrin chain CD18 (Fig. 2 B) or the α chains of LFA-1 (CD11a; Fig. 2 C) or Mac-1 (CD11b; Fig. 2 D). There was no difference between the two genotypes in cell surface staining with a common Fc γ RII/Fc γ RIII-recognizing antibody (Fig. 2 E) or a monoclonal antibody against Fc γ RIV (Fig. 2 F). Collectively, genetic deficiency of PLC γ 2 did not affect neutrophil maturation or the expression of major cell surface integrins or Fc γ receptors.

PLC γ 2 is required for integrin and Fc receptor-mediated neutrophil functions

In the following experiments, the role of PLC γ 2 in *in vitro* neutrophil functions was investigated. Robust neutrophil activation can be achieved by plating the cells on an integrin ligand (e.g., fibrinogen)-coated surface in the presence of a soluble proinflammatory agonist, such as TNF (49), mimicking activation of neutrophils adherent to the extracellular matrix at the site of inflammation. This response is completely dependent on β ₂ integrins both in humans (50) and mice (11) (see Fig. S4). As shown in Fig. 3 A, neutrophils obtained

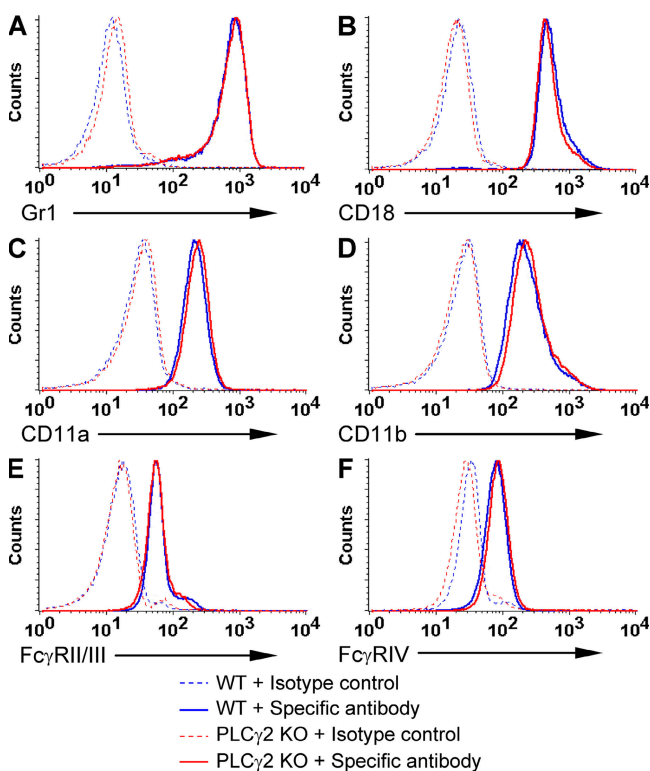


Figure 2. Expression of cell surface molecules on PLC γ 2^{-/-} neutrophils. Expression of the indicated cell surface molecules on unstimulated WT and PLC γ 2^{-/-} (PLC γ 2 KO) bone marrow neutrophils was tested by flow cytometry. Each panel represents three to six independent experiments with similar results.

from PLC γ 2^{-/-} bone marrow chimeras failed to produce superoxide when plated on a fibrinogen-coated surface in the presence of TNF. In a limited number of experiments, a similar defect was also seen in neutrophils isolated from intact (nonchimeric) PLC γ 2^{-/-} mice (Fig. S3 A, available at <http://www.jem.org/cgi/content/full/jem.20081859/DC1>). PLC γ 2^{-/-} neutrophils also failed to release the tertiary granule marker gelatinase (Fig. 3 B) or spread over the fibrinogen surface (Fig. 3 C) under identical conditions. A similar defect was seen when fibrinogen-adherent neutrophils were stimulated by other soluble proinflammatory agents, such as the TLR2 agonist lipopeptide Pam₃CSK₄, the TLR4-specific ligand ultrapurified LPS, the GM-CSF cytokine, or the MIP-2 chemokine, which is the mouse homologue of human IL-8 (Fig. 3 D), in a CD18-dependent manner (Fig. S4).

Although both integrin ligation and a separate proinflammatory stimulus is required for maximal activation of adherent neutrophils under physiological conditions (49, 51), β ₂ integrin-mediated *in vitro* neutrophil activation can also be achieved by plating the cells on surfaces coated with an engineered polyvalent integrin ligand (poly-RGD) in the absence of any additional stimulus (11). PLC γ 2^{-/-} neutrophils failed to release superoxide when plated on a poly-RGD-coated surface (Fig. 3 E) and they did not spread on this polyvalent integrin ligand surface either (Fig. 3 F).

Collectively, PLC γ 2 appears to be critically involved in the adhesion-dependent activation of neutrophils. Together with the fact that both TNF and the other soluble proinflammatory agonists signal normally in PLC γ 2^{-/-} neutrophils in suspension (Fig. 4), our results indicate that PLC γ 2 is required for signaling by integrins rather than by receptors of the soluble proinflammatory agents.

Neutrophils can also be activated by immobilized IgG immune complexes in an Fc γ RIII/Fc γ RIV-dependent manner (46), mimicking their activation upon immune complex deposition in autoimmune diseases. Our unpublished observations indicate that this response also requires Src family kinases and Syk. As shown in Fig. 3 G, neutrophils isolated from PLC γ 2^{-/-} bone marrow chimeras failed to release superoxide when plated on immobilized IgG immune complexes. Similar results were also obtained using neutrophils isolated from intact PLC γ 2^{-/-} mice (Fig. S3 B). The PLC γ 2^{-/-} mutation also abrogated gelatinase release (Fig. 3 H) and neutrophil spreading (Fig. 3 I) under such conditions. Hence, PLC γ 2 is also critically involved in Fc γ receptor-mediated functional responses of neutrophils.

PLC γ 2 is not required for signaling by G protein-coupled receptors, Toll-like receptors, and various cytokine receptors

Next, we tested integrin and Fc receptor-independent responses in PLC γ 2-deficient neutrophils. As a first approach, the cells were stimulated with the nonphysiological protein kinase C-activating agent PMA, which is known to activate neutrophils even in the absence of important cell surface receptors such as β ₂ integrins (11) or intracellular signaling molecules like

Src family kinases (9) or Syk (11). PMA-stimulated $PLC\gamma 2^{-/-}$ neutrophils, which were isolated either from $PLC\gamma 2^{-/-}$ bone marrow chimeras (Fig. 4 A) or intact $PLC\gamma 2^{-/-}$ mice (Fig. S3 C), released normal amounts of superoxide, indicating

that $PLC\gamma 2$ is not required for distal steps of NADPH oxidase activation.

Robust integrin and Fc receptor-independent neutrophil activation can also be triggered by the bacterial formyl-peptide

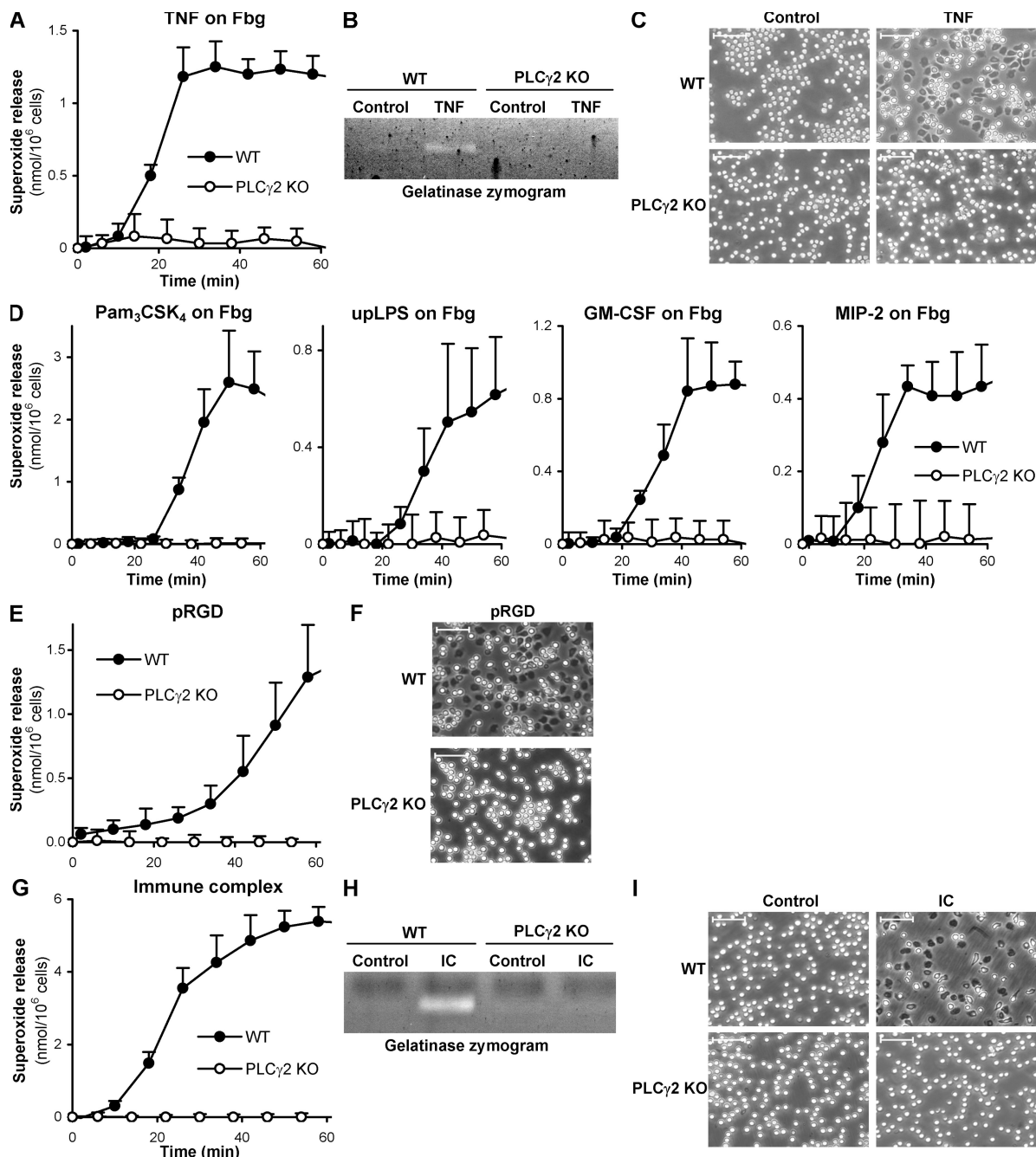


Figure 3. Defective integrin and Fc receptor-mediated responses of $PLC\gamma 2^{-/-}$ neutrophils. (A–C) WT and $PLC\gamma 2^{-/-}$ ($PLC\gamma 2$ KO) neutrophils were activated by 50 ng/ml of murine TNF on a fibrinogen (Fbg)-coated surface and the resulting superoxide production (A), gelatinase release (B), and cell spreading (C) followed. (D) Superoxide release of fibrinogen-adherent neutrophils activated with 1 μ g/ml Pam₃CSK₄, 5 μ g/ml of ultrapurified LPS (upLPS), 10 ng/ml of murine GM-CSF, or 100 ng/ml of murine MIP-2. (E and F) Superoxide release (E) and spreading (F) of neutrophils plated on a polyvalent integrin ligand (poly-RGD)-coated surface (pRGD) in the absence of any additional stimulus. (G–I) Superoxide release (G), degranulation (H), and spreading (I) of neutrophils plated on immobilized IgG immune complexes (IC). Unstimulated control values were subtracted in A, D, and G. Error bars represent SD of triplicate readings. Bars, 50 μ m. Each panel is representative of three to five independent experiments with similar results.

fMLP (which activates G_i protein-coupled receptors), especially if the cells are preincubated with the cytoskeletal disrupting agent cytochalasin B. Under such conditions, fMLP induced similar superoxide production (Fig. 4 B and Fig. S3 D for neutrophils from bone marrow chimeras and intact mice, respectively) and gelatinase release (Fig. 4 C) from WT and $PLC\gamma 2^{-/-}$ cells, indicating that $PLC\gamma 2$ is not required for formyl peptide receptor signal transduction.

In the experiments presented in Fig. 3, adhesion-dependent activation of neutrophils was tested in the presence of various soluble proinflammatory agonists. Because those agonists

do not induce major functional responses (such as respiratory burst) in the absence of an integrin ligand surface (Fig. S4), their integrin-independent signaling capacity was assessed by testing up-regulation of cell surface integrins or activation of intracellular signaling pathways in suspension. TNF triggered normal up-regulation of the CD18 or CD11b integrin chains (Fig. 4 D), normal phosphorylation of the p38 MAP kinase (Fig. 4 E), and normal phosphorylation and degradation of the NF- κ B pathway inhibitor $I\kappa$ -B α (Fig. 4 E) in $PLC\gamma 2^{-/-}$ neutrophils. $PLC\gamma 2$ was not required for phosphorylation of p38 MAP kinase or phosphorylation/degradation of $I\kappa$ -B α

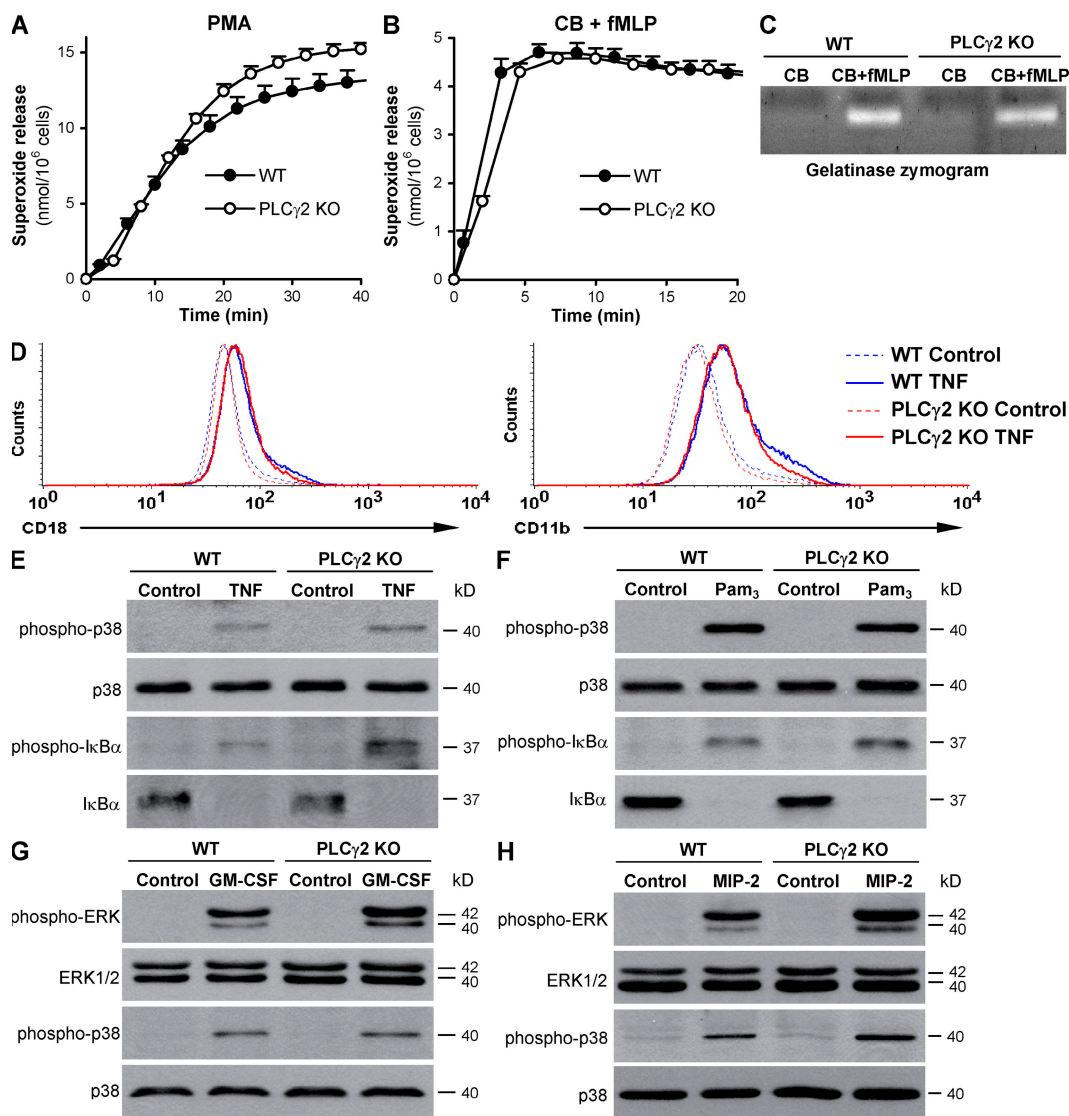


Figure 4. $PLC\gamma 2$ is not required for integrin and Fc receptor-independent neutrophil functions. (A) Superoxide release of WT and $PLC\gamma 2^{-/-}$ ($PLC\gamma 2$ KO) neutrophils stimulated with 100 nM PMA. (B and C) Superoxide production (B) and gelatinase release (C) triggered by 3 μ M fMLP from neutrophils preincubated with 10 μ M cytochalasin B (CB). (D) Up-regulation of CD18 and CD11b upon activation of neutrophils by 50 ng/ml of murine TNF in suspension. (E and F) Phosphorylation of the p38 MAP kinase (p38) and of $I\kappa$ -B α and degradation of $I\kappa$ -B α upon activation of neutrophils with 50 ng/ml of murine TNF (E) or 1 μ g/ml Pam₃CSK₄ (Pam₃; F). (G and H) Phosphorylation of ERK and the p38 MAP kinase upon neutrophil activation by 10 ng/ml of murine GM-CSF (G) or 100 ng/ml of murine MIP-2 (H). Molecular mass values represent the estimated apparent molecular mass of the proteins. Unstimulated controls were subtracted in A and B. Error bars represent SD of triplicate readings. Each panel is representative of three to four independent experiments with similar results.

triggered by the TLR2-specific ligand Pam₃CSK₄ either (Fig. 4 F). Similarly, GM-CSF (Fig. 4 G) and the MIP-2 chemokine (Fig. 4 H) triggered normal ERK and p38 MAP kinase phosphorylation in PLC γ 2^{-/-} neutrophils.

Collectively, PLC γ 2 is not required for integrin and Fc receptor-independent functional and signaling responses of neutrophils. These results also suggest that the defective adherent activation of PLC γ 2^{-/-} neutrophils (Fig. 3) is caused by a defect in integrin signaling rather than that of the soluble proinflammatory agonists.

Normal migration of PLC γ 2-deficient neutrophils

Neutrophil migration to the site of inflammation is mediated by several cell surface receptors including chemokine/chemoattractant receptors and β_2 integrins. Our previous studies indicated that Src family kinases and Syk, which are indispensable for various β_2 integrin-dependent effector functions of neutrophils, are surprisingly not required for β_2 integrin-mediated cell migration (11). Those studies prompted us to test whether PLC γ 2 participates in β_2 integrin-mediated migration of neutrophils.

In an *in vitro* Transwell assay system, PLC γ 2-deficient neutrophils migrated as well as WT cells toward increasing concentrations of the bacterial tripeptide fMLP through a fibrinogen-coated polycarbonate membrane of 3- μ m pore size (Fig. 5 A). Because neutrophil migration under these conditions requires β_2 integrins (11), these results indicate that PLC γ 2 is not required for β_2 integrin-mediated neutrophil migration *in vitro*.

A competitive migration assay during a sterile peritonitis (11) was used to assess the *in vivo* migration of PLC γ 2^{-/-} neutrophils. To this end, mixed bone marrow chimeras carrying both CD45.2-expressing PLC γ 2^{+/+} or PLC γ 2^{-/-} cells, along with CD45.1-expressing PLC γ 2^{+/+} cells in their hematopoietic compartment, were generated. After the induction of a sterile peritonitis by intraperitoneal injection of sterile thioglycollate broth, the percentage of neutrophils from the two donor genotypes was determined both in the bloodstream and the peritoneal infiltrate. Any difference in this percentage between the two compartments would indicate different migratory capacities of neutrophils from the two donor strains. When both CD45.1- and CD45.2-expressing donor cells were of PLC γ 2^{+/+} genotype, the percentage of CD45.2-expressing neutrophils did not differ between the blood and the peritoneum (Fig. 5 B), indicating that the different alleles of CD45 do not affect neutrophil migration. In contrast, when CD45.2-expressing PLC γ 2^{-/-} bone marrow cells and CD45.1-expressing PLC γ 2^{+/+} cells were present the percentage of PLC γ 2^{-/-} cells in the inflamed peritoneum was consistently higher than that in the bloodstream (Fig. 5 B). Calculation of the relative migratory capacity of neutrophils revealed that the accumulation of PLC γ 2^{-/-} neutrophils in the inflamed peritoneum was nearly twice more efficient than that of PLC γ 2^{+/+} cells (Fig. 5 C). These results are in sharp contrast with the severe reduction of migration of CD18^{-/-} neutrophils in a similar assay (11). Therefore, in contrast to

CD18, PLC γ 2 is not required for, or may even act as a negative regulator of, neutrophil migration into the inflamed peritoneum. Collectively, these results indicate that, similar to Src family kinases and Syk, PLC γ 2 is not required for CD18-dependent *in vitro* or *in vivo* migration of neutrophils.

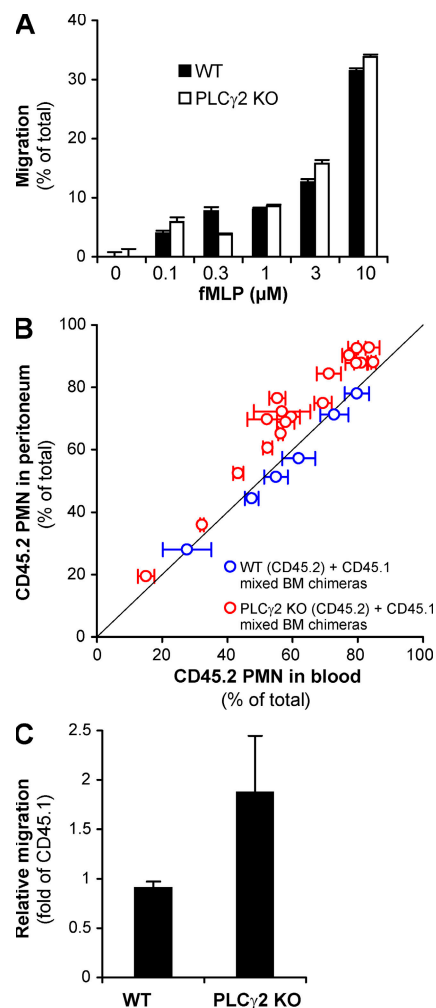


Figure 5. Normal *in vitro* and *in vivo* migration of PLC γ 2^{-/-} neutrophils. (A) Migration of WT and PLC γ 2^{-/-} (PLC γ 2 KO) neutrophils toward the indicated concentrations of fMLP through fibrinogen-coated transwell membranes of 3- μ m pore size. Error bars represent SD of duplicate readings. Data are representative of three independent experiments. (B and C) Competitive migration of CD45.2-expressing and CD45.1-expressing neutrophils during thioglycollate-induced sterile peritonitis in mixed bone marrow chimeras. (B) Percentage of CD45.2-expressing WT or PLC γ 2^{-/-} cells in the blood and the peritoneal lavage fluid. Each data point represents an individual mouse. The thin diagonal line marks points of identical percentage of CD45.2 cells in the blood and the peritoneum. Error bars represent SD from three blood samples taken at different time points from the same mouse. The data are combined from two independent experiments. (C) Relative migratory capacity of CD45.2-expressing WT or PLC γ 2^{-/-} neutrophils relative to the CD45.1-expressing cells calculated from the data presented in B. Error bars represent SD of values from 6 (WT) or 18 (PLC γ 2^{-/-}) individual mice.

Leukocyte–endothelial interaction in fMLP-treated cremaster muscles in vivo

To gain further insight into the relationship between spreading, adherent activation, and cell migration in neutrophils, as well as to exclude the possibility that the aforementioned differences between the role of PLC γ 2 in these processes (compare Figs. 3 and 5) stem from the very different assay systems used, we performed the simultaneous analysis of leukocyte adhesion, spreading, and extravasation in individual venules of fMLP-superfused cremaster muscles of WT and PLC γ 2^{-/-} bone marrow chimeras. As shown in Table S1 (available at <http://www.jem.org/cgi/content/full/jem.20081859/DC1>), there was no difference in the various hemodynamic parameters or total leukocyte counts between the two genotypes. The PLC γ 2^{-/-} mutation did not affect rolling flux fraction (36 ± 11 and $28 \pm 12\%$ in WT and PLC γ 2^{-/-} chimeras, respectively) or leukocyte adhesion (Fig. 6 A) under resting conditions either. However, although local superfusion of the cremaster muscle of WT chimeras with 1 μ M fMLP triggered a significant increase in stable adhesion of leukocytes

to the vessel wall, no such effect was observed in PLC γ 2^{-/-} chimeras (Fig. 6 A). fMLP also induced the spreading of WT leukocytes, as indicated by the flattening (decreased diameter perpendicular to the vessel wall) of the cells adherent to the endothelium (Fig. 6 B). This spreading (flattening) response was strongly reduced in PLC γ 2^{-/-} bone marrow chimeras at early time points (Fig. 6 B), although the mutant cells were able to partially flatten down at later time points after fMLP stimulation. Besides these real-time in vivo microscopic observations, parallel cremaster muscle samples were subjected to whole mount histological analyses. Those studies again revealed that the fMLP-induced increase of the intravascular leukocyte count (an approximate measure of leukocyte adhesion) was significantly attenuated in PLC γ 2^{-/-} bone marrow chimeras (Fig. 6 C), likely reflecting the described adhesion/spreading defect (Fig. 6, A and B). Despite all these observations, the fMLP-induced increase of the number of perivascular leukocytes (an approximate measure of leukocyte extravasation) in PLC γ 2^{-/-} chimeras was similar to or even slightly higher than that in WT control chimeras (Fig. 6 D),

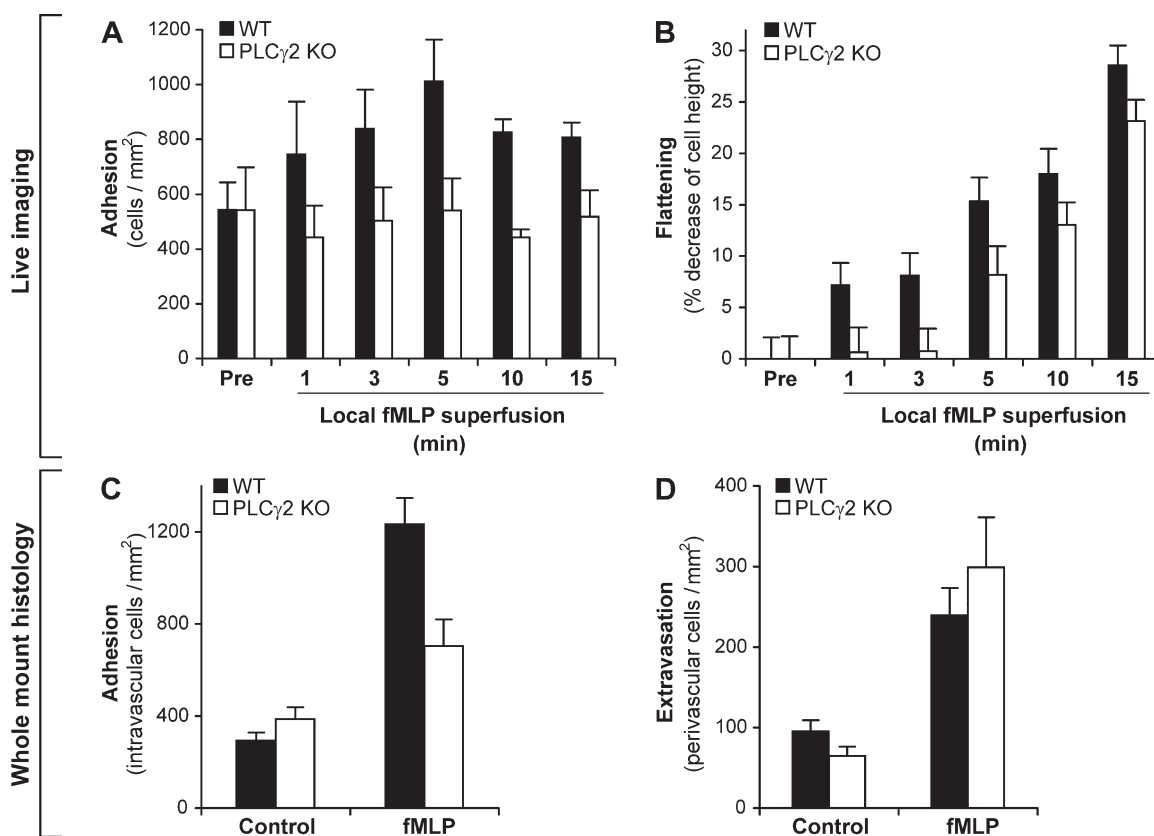


Figure 6. Leukocyte–endothelial interaction in fMLP-treated cremaster muscle venules in vivo. (A and B) Intravital microscopy of postcapillary cremaster muscle venules superfused with 1 μ M fMLP. (A) Leukocyte adhesion in postcapillary venules of WT and PLC γ 2^{-/-} (PLC γ 2 KO) bone marrow chimeras before (pre) and at the indicated time points during superfusion with fMLP. (B) Leukocyte spreading in fMLP-superfused cremaster muscle venules. The rate of spreading is expressed as the percent decrease in cell diameter perpendicular to the vessel wall. Mean and SEM of data obtained from four WT and five PLC γ 2 KO chimeras are shown. (C and D) Leukocyte adhesion (C) and extravasation (D) assessed by histological analysis of whole mount preparations of cremaster muscles of WT or PLC γ 2 KO bone marrow chimeras superfused for 15 min in the presence or absence of 1 μ M fMLP. The mean and SEM are shown of the number of intravascular (C) and perivascular (D) leukocytes in 29–41 individual vessels per group from four WT and five PLC γ 2 KO chimeras, each tested independently during the same day.

suggesting that transendothelial migration of leukocytes was not impaired in the absence of PLC γ 2. These results again indicate that a defective adhesion/spreading response in the absence of PLC γ 2 does not translate into impaired migration of leukocytes through the vessel wall.

PLC γ 2^{-/-} bone marrow chimeras are protected from macroscopic and microscopic signs of autoimmune arthritis

The role of PLC γ 2 in integrin and Fc receptor-mediated neutrophil functions raise the possibility that PLC γ 2 may be

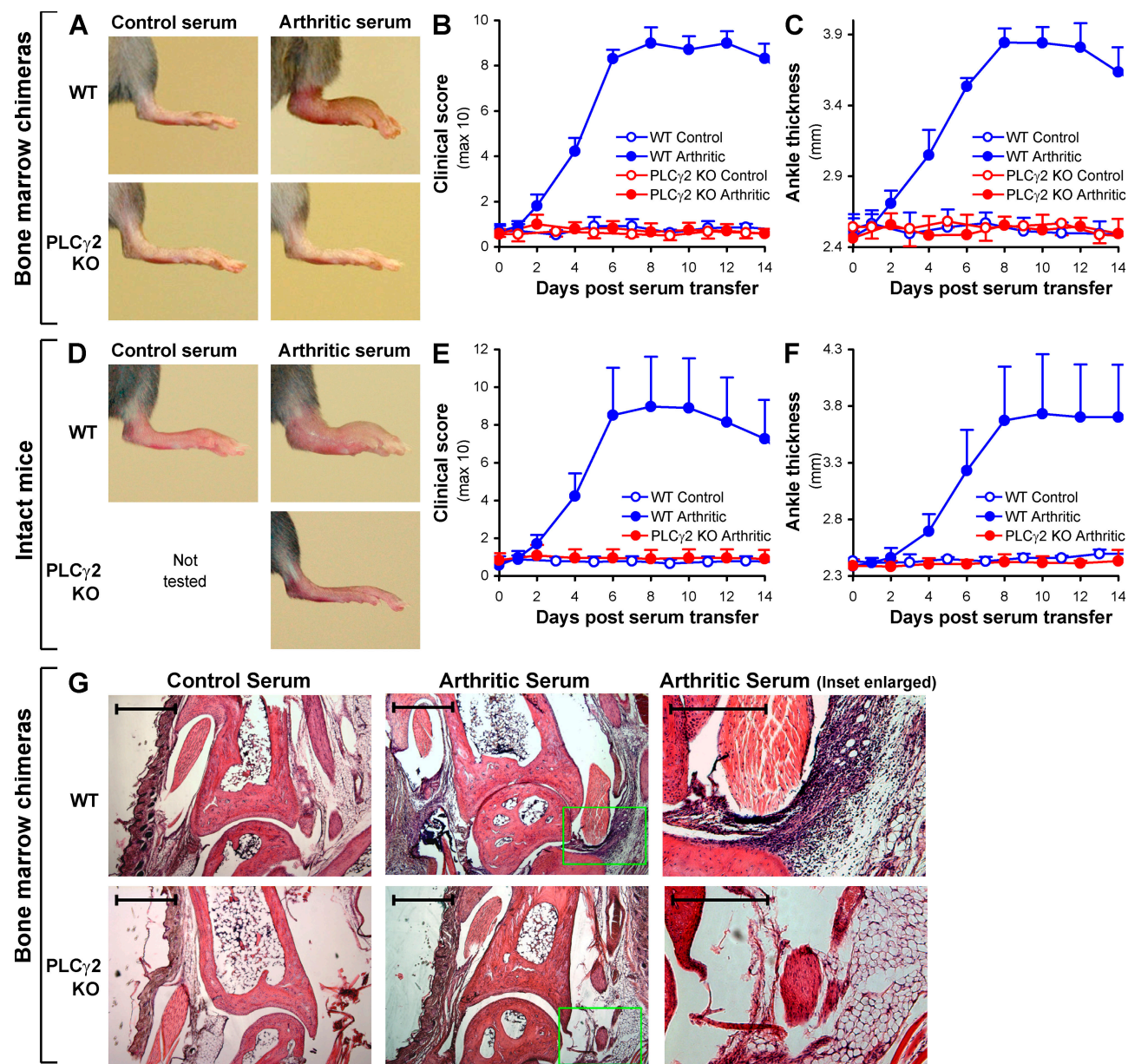


Figure 7. PLC γ 2 is required for the development of K/BxN serum transfer arthritis. WT and PLC γ 2^{-/-} (PLC γ 2 KO) bone marrow chimeras (A–C and G) or intact (nonchimeric) mice (D–F) were injected with 400 μ l of arthritic (K/BxN) or nonarthritic control serum and the development of arthritis followed. (A) Photographs of the hind limb of mice of the indicated treatment and hematopoietic genotype 10 d after serum injection. Pictures are representative of a total of 17–23 individual mice per group from eight independent experiments. (B and C) Hind limb clinical score (B) and ankle thickness (C) of mice of the indicated treatment and genotype. Error bars represent the SD of four to eight individual clinical scores or ankle thickness values from a single experiment repeated a total of eight times. (D–F) Hind limb photographs (D), clinical score (E), and ankle thickness (F) of intact (nonchimeric) mice of the indicated treatment and genotype. Data are from three mice per group tested in parallel. Error bars represent the SD of six individual hind limb values from three mice per group. (G) Histological analysis of the ankle joint of mice of the indicated treatment and hematopoietic genotype 4 d after serum injection. The photomicrographs on the right are enlarged from the highlighted areas in the middle pictures. Original magnification, 5 \times . Bars: (left and middle) 200 μ m; (right) 100 μ m. Photomicrographs are representative of a total of four to six samples per group from three independent experiments.

involved in the pathogenesis of inflammatory diseases mediated by these factors. To test this possibility, we turned to the K/B×N serum transfer arthritis model, an autoantibody-mediated model of the effector phase of autoimmune arthritis. Prior studies from other groups indicated that this model requires neutrophils (27, 32, 33) as well as the presence of β_2 integrins (44) and Fc γ receptors (34–41). We have also confirmed the latter two conclusions (unpublished data).

To test the role of PLC γ 2 in the K/B×N serum transfer arthritis model, WT or PLC γ 2^{-/-} bone marrow chimeras were injected with arthritogenic K/B×N serum or normal serum from nonarthritic (KRN transgene negative) littermates. Although WT bone marrow chimeras injected with arthritogenic serum developed severe arthritis of their hind paws (Fig. 7 A), no sign of the disease was seen in similarly

treated PLC γ 2^{-/-} chimeras (Fig. 7 A), indicating a major role for PLC γ 2 in the development of K/B×N serum transfer arthritis. Quantification of arthritis severity by clinical scoring revealed that arthritis became evident 2 d after injection of WT chimeras with arthritogenic serum, peaked between 8–12 d, and started to cease afterward. Importantly, no signs of arthritis were seen at any time point in PLC γ 2^{-/-} bone marrow chimeras injected with arthritogenic K/B×N mouse serum (Fig. 7 B). Treatment of WT chimeras with arthritogenic serum also triggered a robust increase of their ankle thickness (Fig. 7 C), whereas the same treatment had no effect on ankle thickness of PLC γ 2^{-/-} chimeras (Fig. 7 C). Collectively, PLC γ 2 within the hematopoietic compartment is indispensable for the development of macroscopic signs of autoimmune arthritis in the K/B×N serum transfer model.

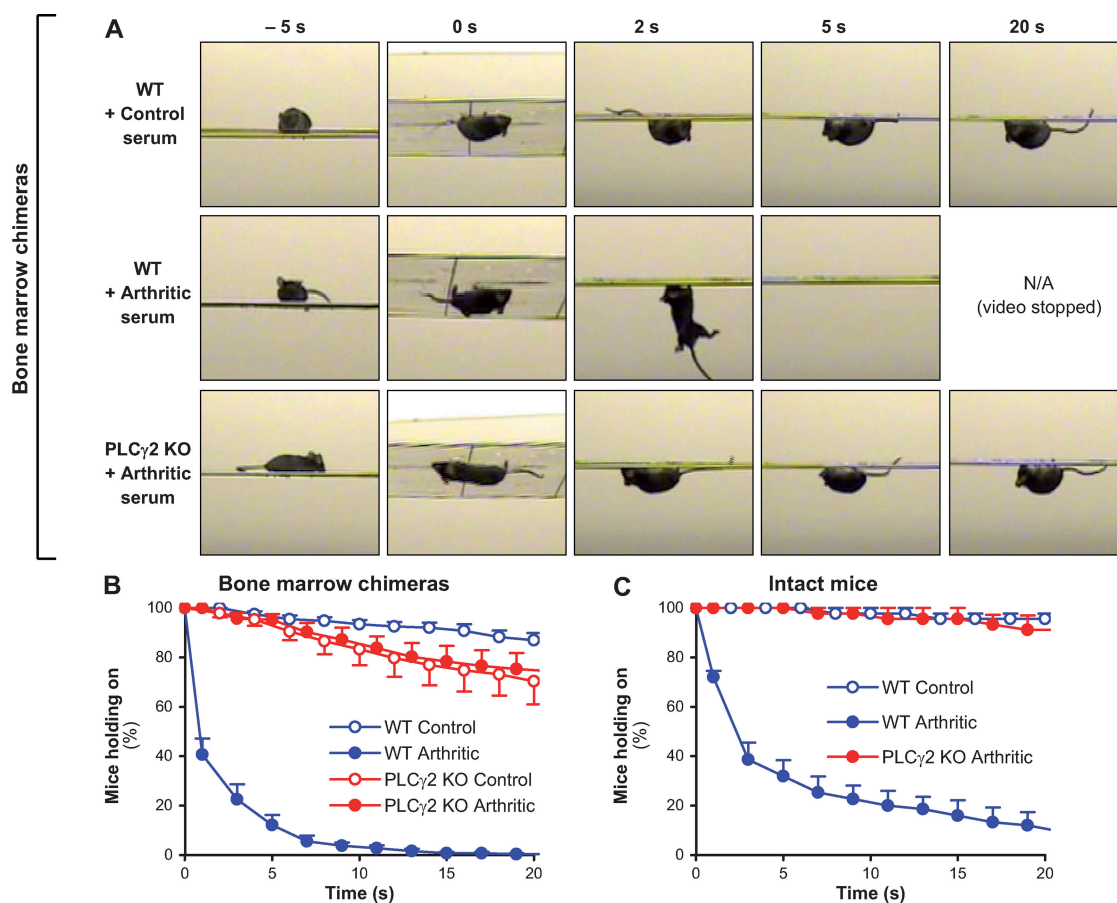


Figure 8. PLC γ 2 deficiency protects from arthritis-induced loss of articular function. WT and PLC γ 2^{-/-} (PLC γ 2 KO) bone marrow chimeras (A and B) or intact (nonchimeric) mice (C) were injected with 400 μ l of arthritic (K/B×N) or nonarthritic control serum. 6–12 d after the serum injection, the mice were placed on a custom-made wire grid, flipped over, and the time for which the mice were able to hold on to the lower side of the grid was recorded. (A) Snapshots at the indicated time points from video captures of mice of the indicated treatment and hematopoietic genotype 10 d after serum injection. The snapshots are representative of a total of 165–263 individual measurements on 10–16 mice per group from four independent experiments. (B) Quantitative analysis of the articular function as represented by the percentage of the bone marrow chimeras from a given group to hold on to the grid for a given period of time after the grid has been flipped over from four independent experiments. Error bars represent SEM of 10–16 individual “holding on curves” (obtained from 12–21 measurements on each single mouse between 8 and 12 d after serum transfer). (C) Quantitative analysis of the articular function of intact mice of the indicated treatment and genotype. Error bars represent SEM of three individual holding on curves (obtained from 18 measurements on each single mouse between 8 and 12 d after serum transfer).

To exclude the possibility that these results were affected by the bone marrow transplantation approach (e.g., by the use of irradiation that by itself may affect the course of autoimmune arthritis [reference 52]), the same experiments were repeated on a small cohort of intact (nonchimeric) WT and PLC γ 2^{-/-} mice. As shown in Fig. 7 (D–F), genetic deficiency of PLC γ 2 completely abrogated the development of all clinical signs of K/B \times N serum transfer arthritis even in such nonchimeric animals.

We also performed histological analysis of the ankle joint of bone marrow chimeras of the various experimental groups. As shown in Fig. 7 G, a robust leukocytic infiltration of the periarticular tissues could be observed in WT chimeras injected with arthritogenic serum relative to those injected with nonarthritogenic control serum. Importantly, no such infiltration was seen in PLC γ 2^{-/-} bone marrow chimeras injected with arthritogenic serum (Fig. 7 G), indicating that PLC γ 2 is required for the development of microscopic signs of arthritis such as the accumulation of leukocytes in the periarticular space.

PLC γ 2^{-/-} bone marrow chimeras are protected from arthritis-induced loss of articular function

Besides the macroscopic and microscopic signs of inflammation, arthritis also leads to severe impairment of articular function. This was assessed by testing the ability of the mice to hold on to the bottom of a horizontal wire grid similar to a regular wire cage lid. As shown in the video snapshots in Fig. 8 A, although WT chimeras injected with control serum were able to hold on to the wire grid for the entire 20-s assay period, WT chimeras injected with arthritogenic serum were not able to hold on for more than a few seconds, indicating an arthritis-induced loss of articular function. Importantly, PLC γ 2^{-/-} bone marrow chimeras injected with arthritogenic serum had no difficulties in holding on to the wire grid for the entire assay period (Fig. 8 A).

To obtain a more quantitative assessment of joint function, this experiment was repeated several times on each individual mouse during the plateau phase of the disease, and the percentage of mice that were still holding on to the wire grid at a given time point was calculated analogous to Kaplan-Meier survival curves (Fig. 8 B). As shown in Fig. 8 B, nearly 90% of control-treated WT chimeras held on to the wire grid until the end of the 20-s assay period. In contrast, only 40% of WT chimeras injected with arthritogenic serum held on for >1 s, and practically none of them did so for the entire assay period (Fig. 8 B). Importantly, most of the PLC γ 2^{-/-} chimeras were able to hold on to the wire grid for the entire 20-s period irrespective of whether they were injected with arthritogenic or control serum (Fig. 8 B). In a small set of experiments, a similar protection from arthritis-induced loss of articular function was seen in intact (nonchimeric) PLC γ 2^{-/-} mice (Fig. 8 C), indicating that the effect of PLC γ 2 deficiency on articular function was not affected by the bone marrow transplantation approach used. Collectively, mice lacking PLC γ 2 are also protected from arthritis-induced loss of articular function.

DISCUSSION

Rheumatoid arthritis is a severe chronic disease affecting ~1% of the human population. Although the therapy of the disease has significantly improved during the last decades, it is still far from being solved. This is exemplified by the still widespread use of the highly cytotoxic chemotherapeutic agent methotrexate, the severe cardiovascular complications of COX-2 inhibitors, or the tremendous costs and possible side effects (e.g., reactivation of silent tuberculosis) of anti-TNF therapeutics. Better understanding of rheumatoid arthritis at the molecular level would strongly facilitate the development of novel treatment strategies for the disease.

The experiments presented in this paper provide evidence for the role of PLC γ 2 in the K/B \times N arthritis model, one of the most widely used animal models of rheumatoid arthritis. A unique feature of this model is that its effector phase can be clearly separated from its initiation phase by transferring the serum of an arthritic K/B \times N mouse to an otherwise nonarthritic recipient (26). Our experiments performed using this serum transfer model (Figs. 7 and 8) indicate that PLC γ 2 participates in the effector phase of the disease. Furthermore, the fact that bone marrow chimeras with PLC γ 2^{-/-} hematopoietic system but PLC γ 2^{+/+} nonhematopoietic tissues are protected from K/B \times N serum transfer arthritis indicates that PLC γ 2 within the hematopoietic system is indispensable for disease development.

The effector phase of rheumatoid arthritis is mediated by several cell types, likely including various phagocytic lineages. To our knowledge, of those lineages only neutrophils have been consistently linked to the development of the inflammatory process in a diverse array of arthritis models and experimental approaches (27–33). Of the other phagocytes, liposome-mediated depletion studies suggested a pathogenetic role for macrophages (53), but another genetic study indicated that certain macrophage subsets play a negative rather than a positive role in autoimmune arthritis (54). Although an elegant series of genetic and reconstitution studies indicated the role of mast cells in the development of K/B \times N serum transfer arthritis (55), another study using a different genetic approach suggested that arthritis development may proceed normally in the absence of mast cells (56). Based on these results, the most likely explanation for our *in vivo* results is that PLC γ 2 within neutrophils is required for the autoantibody-induced inflammation process. The second most likely PLC γ 2-dependent compartment would be the mast cell lineage, which also expresses PLC γ 2 and may be activated through integrins and Fc γ receptors. However, because mast cells are long-lived radioresistant cells that survive a lethal irradiation in most tissues (57, 58), it is unlikely that our bone marrow transplantation approach was able to replace the majority of the recipients' mast cells. Hence, it is unlikely that the complete defect of arthritis development in PLC γ 2^{-/-} bone marrow chimeras is caused solely by the deficiency of PLC γ 2 in mast cells.

Several cell surface receptors have also been shown to participate in various models of autoimmune arthritis. Several

studies using mice lacking the Fc receptor common γ chain (34–37) or Fc γ receptor-specific ligand binding α chains (36–42) indicated a critical role for Fc γ receptors in various autoimmune arthritis models (43). The role of β_2 integrins (44) or their putative ligands (44, 45) has also been shown in various autoimmune arthritis models. Somewhat surprisingly, however, there is very little information available on whether and to what extent signaling molecules downstream of these receptors play a role in the development of autoimmune arthritis. In this context, it is particularly important that our study identifies PLC γ 2, a component of integrin and Fc receptor signal transduction, as a critical player of the effector phase of autoimmune arthritis.

Neutrophils are critical players of the innate immune response but they also participate in tissue destruction during autoimmune diseases (1–3, 5). Integrins and Fc receptors are two major groups of cell surface receptors participating in neutrophil activation at the site of inflammation or bacterial invasion. We and others have shown that integrin and Fc receptor signaling in neutrophils are both mediated by a receptor-proximal tyrosine phosphorylation cascade consisting of Src family kinases, ITAM-bearing adaptor molecules, and the Syk tyrosine kinase (6–11; for review see reference 16) (unpublished data). In the present work, we identify PLC γ 2 as a common downstream mediator of integrin and Fc receptor signaling in neutrophils (Fig. 3). PLC γ 2 thus appears to be a new member of a growing family of intracellular molecules participating in both integrin and Fc receptor signal transduction in these cells (6–11, 59–61; for review see reference 16). In contrast, PLC γ 2 is dispensable for neutrophil activation through several other cell surface receptors such as G protein-coupled formyl peptide or chemokine receptors, various cytokine receptors (TNF and GM-CSF), or members of the Toll-like receptor family (Fig. 4). Hence, PLC γ 2 plays a specific role in signaling by a defined subset of neutrophil activatory receptors.

In addition to participating in adhesion-dependent functional responses of neutrophils, β_2 integrins are also required for the migration of the cells to the site of inflammation. Somewhat surprisingly, although PLC γ 2 is required for the former response (Fig. 3), β_2 integrin-mediated (11) neutrophil migration can occur in the absence of PLC γ 2 (Fig. 5). This is, however, in line with the normal migration of neutrophils lacking Src family kinases (11), Syk (11), ITAM-bearing adaptors (10), or members of the Vav family (60) under CD18-dependent conditions (11). Therefore, β_2 integrins likely use other PLC γ 2-independent pathways to support neutrophil migration to the site of inflammation.

It is also puzzling how and to what extent spreading and extravasation can be dissected at the cellular and molecular level, given the generally accepted view that spreading and firm leukocyte adhesion precedes the transmigration of leukocytes through the vessel wall. Our prior studies on leukocyte-endothelial interactions in Syk $^{-/-}$ bone marrow chimeras (62, 63) indicated that a significant level of extravasation is possible even when adhesion is severely reduced and leukocyte spreading over the endothelium is apparently completely

absent. Similar studies on PLC γ 2 $^{-/-}$ bone marrow chimeras presented in this paper also indicate that severely defective adhesion (Fig. 6, A and C), and decreased and delayed spreading (Fig. 6 B) do not necessarily hinder the extravasation (Fig. 6 D) of leukocytes. Hence, transmigration of leukocytes through the vessel wall is possible even if adhesion and spreading are severely defective. It is at present unclear how transendothelial migration occurs under these conditions. One possibility is that integrin-mediated spreading and firm adhesion simply coincide with concomitant transmigration without any major role of the former two processes in the latter one. Alternatively, spreading and adhesion may play dual roles by promoting transmigration, for example, through arresting the leukocytes at the site of inflammation, but also hindering it, for example, by holding leukocytes back by the adhesive process. If so, then a defective adhesion/spreading response would not have any major net effect on transmigration of leukocytes. Further studies will be required to reveal whether and how these or other mechanisms can explain transmigration of leukocytes when their spreading and/or adhesion responses are severely impaired.

Along the same line of thinking, it is also interesting to note that our histological analyses showed a complete lack of leukocytic infiltration in periarticular regions of PLC γ 2 $^{-/-}$ bone marrow chimeras injected with arthritogenic K/B \times N serum (Fig. 7 G). Based on the normal migration of PLC γ 2 $^{-/-}$ neutrophils under other conditions (Figs. 5 and 6), we hypothesize that the lack of PLC γ 2 blocks the development of the inflammatory environment (chemokines, cytokines, and inflammatory endothelium). Hence, the otherwise migration-competent neutrophils are not attracted to the periarticular tissues in PLC γ 2 $^{-/-}$ bone marrow chimeras.

All of the experiments presented in this paper have been performed on inbred mice on the C57BL/6 genetic background. This strain is the most widely used genetically homogeneous mouse strain, allowing the most accurate comparison of our results with those from other investigators. However, we cannot exclude the possibility that PLC γ 2 would be less critically involved in *in vitro* neutrophil functions and/or the effector phase of autoimmune arthritis on a different genetic background. Such a phenomenon could theoretically be possible through compensation by either PLC γ 1 or a PLC γ -independent mechanism. Although it would be rather difficult to predict the possible extent of compensation by the latter mechanism, studies showing that overexpression of PLC γ 1 in PLC γ 2 $^{-/-}$ cells was not able to restore B cell maturation (64) or the development of multinucleated osteoclasts (65) suggest that there is relatively little room for functional compensation between the two members of the PLC γ family.

In addition to providing clear evidence for a role of PLC γ 2 in integrin-mediated neutrophil functions and the development of K/B \times N serum transfer arthritis, our studies also raise several novel questions that have yet to be addressed in the future. Although we hypothesize that the effect of the PLC γ 2 $^{-/-}$ mutation *in vivo* is a result of a neutrophil defect, this has yet to be confirmed more directly, for example, by

lineage-specific deletion of PLC γ 2 in neutrophils. The same holds true for whether our *in vivo* phenotype is indeed caused by an integrin and/or Fc receptor signaling defect and whether PLC γ 2^{-/-} neutrophils are indeed capable of migrating to the site of inflammation during a full-blown K/B \times N serum transfer arthritis. Although our studies strongly implicate PLC γ 2 in the effector phase of autoantibody-mediated arthritis, its contribution to other aspects of the disease have yet to be tested, for example, using the TNF-mediated human TNF-transgenic Tg197 (66) or the IL-17-mediated SKG point mutant (67, 68) models. It is also unclear whether the lipase activity and/or other structural features of PLC γ 2 contribute to its role in neutrophil functions *in vitro* and arthritis development *in vivo*. This, and the reason for why PLC γ 1 is apparently not able to compensate for the lack of PLC γ 2, will need to be tested by reexpression of various PLC γ 2 mutants and/or PLC γ 1 in PLC γ 2^{-/-} bone marrow cells, for example, by using a retroviral reconstitution strategy (10). Finally, the role of PLC γ 2 in the various aspects of antimicrobial functions of leukocytes (such as phagocytosis by myeloid lineage cells) and its relationship to that of Src family kinases and Syk has yet to be tested in more detail.

Collectively, we found that PLC γ 2 is a central component of integrin and Fc receptor signal transduction in neutrophils, linking the receptor-proximal Src family-ITAM adaptor-Syk cascade to functional responses of these cells. Our results also identify PLC γ 2 as a critical player of the effector phase of autoimmune arthritis, most likely through its role in integrin and Fc receptor signaling of neutrophils. These studies provide novel insight into the cellular and molecular mechanisms of autoimmune inflammation and may eventually point to novel targets of future therapies of major human diseases such as rheumatoid arthritis.

MATERIALS AND METHODS

Animals. Heterozygous mice carrying a deleted PLC γ 2 allele (PLC γ 2^{tm1Jm}, referred to as PLC γ 2^{-/-}) (19) were obtained from J. Ihle (St. Jude Children's Research Hospital, Memphis, TN). Because of the limited fertility and survival of homozygous PLC γ 2^{-/-} mice, the mutation was maintained in heterozygous form by a PLC γ 2^{+/-} \times PLC γ 2^{+/-} breeding strategy. Offsprings were genotyped by allele-specific PCR reaction from tail DNA using 5'-GCCTCTGCACAGCACACATATGG-3' WT-specific and 5'-CAA-GGTGAGATGACAGGAGATCC-3' mutant-specific forward primers along with the 5'-TTCACCGCATCTCTTTGAGTCC-3' common reverse primer. Triple Src family-deficient (*Hck*^{tm1Hev/tm1Hev}*Fgr*^{tm1Hev/tm1Hev}*Lyn*^{tm1Sor/tm1Sor}, referred to as *Hck*^{-/-}*Fgr*^{-/-}*Lyn*^{-/-}) mice (69) were obtained from C. Lowell (University of California, San Francisco, San Francisco, CA) and kept as triple homozygous mutants. Mice carrying the *Syk*^{tm1Tyb} mutation (70) (referred to as the *Syk*⁻ allele) were obtained from V. Tybulewicz (National Institute for Medical Research, London, UK). The *Syk*⁻ mutation was maintained in heterozygous form and used to obtain *Syk*^{-/-} neutrophils by fetal liver transplantation as previously described (11). Mice carrying the KRN T cell receptor transgene (25) were obtained from D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) and maintained in heterozygous form by mating with C57BL/6 mice. KRN transgene-positive mice were identified by flow cytometry (see Flow cytometry) based on the high percentage of V β 6 TCR-expressing cells among CD4-positive T cells (25). Complete CD18-deficient (*Itgb*^{2m2Bay/tm2Bay}, referred to as CD18^{-/-}) mice (71) were obtained from A. Beaudet (Baylor College of Medicine, Hous-

ton, TX). Fc receptor γ chain-deficient (*Fcer1g*^{tm1Rav/tm1Rav}, referred to as *FcR γ* ^{-/-}) mice (72) were purchased from Taconic. All these mice were backcrossed to the C57BL/6 genetic background for eight or more generations. WT control C57BL/6 mice were purchased from the Hungarian National Institute of Oncology. NOD mice, as well as a congenic strain carrying the CD45.1 allele on the C57BL/6 genetic background (B6.SJL-*Ptprc*^e), were purchased from The Jackson Laboratory. Mice were kept in individually sterile ventilated cages (Tecniplast) in a conventional facility. All animal experiments were approved by the Semmelweis University (Budapest, Hungary) Animal Experimentation Review Board or the Regierungspräsidentium Karlsruhe (Karlsruhe, Germany).

To obtain bone marrow chimeras with PLC γ 2^{-/-} hematopoietic system, recipients carrying the CD45.1 allele on the C57BL/6 genetic background were lethally irradiated by 11 Gy from a ⁶⁰Co source using an irradiator (Gammatron 3; Siemens) and then injected intravenously with unfractionated bone marrow cells from PLC γ 2^{-/-} or WT C57BL/6 control mice. On average, bone marrow cells of a single donor mouse were injected into 10–12 recipients. 4–6 wk after transplantation, peripheral blood samples were stained for Gr1 and CD45.2 and analyzed by flow cytometry (see Flow cytometry). Repopulation of the hematopoietic compartment by donor-derived cells was defined as the percentage of CD45.2-positive (donor-derived) cells in the Gr1-positive granulocyte gate. Bone marrow chimeras were used 5–10 wk after the transplantation.

Neutrophil isolation. Mouse neutrophils were isolated from the bone marrow of the femurs and tibias by hypotonic lysis followed by Percoll (GE Healthcare) gradient centrifugation as previously described (73). Neutrophil isolation was performed at room temperature using sterile and endotoxin-free reagents. Cells were kept at room temperature in Ca²⁺- and Mg²⁺-free medium until use (usually less <30 min) and prewarmed to 37°C before activation. Neutrophil assays were performed at 37°C in Hank's balanced salt solution (Invitrogen) supplemented with 20 mM Hepes, pH 7.4.

Flow cytometry. For neutrophil studies, isolated neutrophils, peripheral blood samples, or peritoneal lavage fluids were stained with PE-conjugated anti-Gr1 (RB6-8C5), FITC-conjugated anti-CD45.2 (clone 104), biotinylated anti-CD11b (M1/70), or unconjugated antibodies against CD18 (C71/16), CD11a (M17/4), Fc γ RII/III (2.4G2), or Fc γ RIV (9E9; obtained from J. Ravetch, Rockefeller University, New York, NY) (74). To identify KRN transgene-positive mice, peripheral blood samples were labeled with FITC-conjugated anti-CD4 (RM4-5) and PE-conjugated anti-TCR V β 6 (RR4-7) antibodies. Unconjugated antibodies were visualized with FITC-conjugated anti-rat IgG whereas biotinylated anti-CD11b was visualized with streptavidin-Cy3 (Jackson ImmunoResearch Laboratories). Unless otherwise stated, all flow cytometry antibodies and their isotype controls were purchased from BD. All staining was performed in the presence of 2% FCS (Invitrogen). Samples were fixed in FACS Lysing Solution (BD) and analyzed on a FACSCalibur (BD) using CellQuest software (BD). Neutrophils and CD4-positive T cells were identified based on positive labeling for Gr1 and CD4, respectively, along with their typical forward- and side-scatter characteristics.

In vitro functional assays. Adhesion-dependent activation was performed by stimulating murine neutrophils with 50 ng/ml of recombinant murine TNF (PeproTech), 1 μ g/ml Pam₃CSK₄ (EMC Microcollections), 5 μ g/ml of ultrapurified LPS (InvivoGen), 10 ng/ml of recombinant murine GM-CSF (PeproTech), or 100 ng/ml of recombinant murine MIP-2 (PeproTech) while adherent to a plastic surface coated with 150 μ g/ml of human fibrinogen (MP Biomedicals) as previously described (10, 11). Integrin-mediated activation in the absence of another soluble stimulus was achieved by plating neutrophils on surfaces precoated with 20 μ g/ml of engineered polyvalent integrin ligand peptide (poly-RGD; F5022; Sigma-Aldrich). Neutrophil activation by immobilized immune complexes was achieved by plating the cells on immobilized HSA-anti-HSA (both obtained from Sigma-Aldrich) immune complexes without any additional stimulus as previously described (46). Activation of neutrophils in suspension was performed in Mg²⁺-free

media essentially as previously described (10, 11, 73, 75) using 100 nM PMA (Sigma-Aldrich), 3 μ M fMLP (Sigma-Aldrich), or 50 ng/ml TNF. fMLP-stimulated cells were pretreated with 10 μ M cytochalasin B (CB; Sigma-Aldrich) for 10 min before cell activation. Where necessary, the reaction was stopped after 10 min (degranulation triggered by CB+fMLP) or 30 min (integrin and Fc receptor-mediated degranulation and spreading responses; TNF-induced integrin up-regulation).

Superoxide release was determined by a real-time cytochrome *c* (Sigma-Aldrich) reduction test, as previously described (51), using a multiplate reader (Multiskan Ascent; Thermo Fisher Scientific) in dual wavelength (550 and 540 nm) kinetic measurement mode. To simplify the presentation, unstimulated control values were subtracted from those of stimulated samples. Exocytosis of gelatinase was determined by in-gel gelatinase zymography as previously described (10, 46). Cell spreading was assessed after formalin fixation using an inverted microscope (DMI 6000B; Leica) with a 20 \times phase-contrast objective connected to a charge-coupled device camera (DFC480; Leica).

Biochemical and signaling studies. Unstimulated WT and PLC γ 2^{-/-} neutrophils were lysed in a 1% Triton X-100-based lysis buffer (11, 73), and their Triton-soluble fraction were boiled in sample buffer, run on SDS-PAGE, and immunoblotted with antibodies against PLC γ 2 (Q-20; Santa Cruz Biotechnology, Inc.), PLC γ 1 (1249; Santa Cruz Biotechnology, Inc.), or β -actin (AC-74; Sigma-Aldrich), followed by peroxidase-labeled secondary antibodies (GE Healthcare). Where indicated, primary antibodies were preincubated with 0.4 μ g/ml of the relevant blocking peptides (Santa Cruz Biotechnology, Inc.) before incubation with the immunoblotting membrane. Lysates prepared from thymus or spleen cells of WT mice served for comparisons of signal intensity.

Purified recombinant Myc-tagged human PLC γ 1 and PLC γ 2, expressed in Sf9 insect cells using a baculoviral expression system, were obtained from P. Gierschik (University of Ulm, Ulm, Germany) (76). For the quantification (titration) of PLC γ 1 and PLC γ 2 expression in neutrophils, various amounts of the two recombinant proteins along with murine neutrophil lysates were run on SDS-PAGE and immunoblotted using the aforementioned PLC γ 1 and PLC γ 2 antibodies as well as an antibody against the Myc epitope (clone 9E10; Santa Cruz Biotechnology, Inc.). The amino acid sequences of the human and murine proteins are practically identical around the putative antibody recognition sites in the cases of both PLC γ isoforms, whereas there is hardly any similarity between PLC γ 1 and PLC γ 2 at the same sites, which justifies the use of recombinant human PLC γ isoforms along with the aforementioned polyclonal antibodies for the quantification of the two murine proteins.

For biochemical signaling experiments, neutrophils were plated on a poly-RGD or immobilized immune complex surface, or they were stimulated by 50 ng/ml TNF, 1 μ g/ml Pam₃CSK₄, 10 ng/ml GM-CSF, or 100 ng/ml MIP-2 in Mg²⁺-free media in suspension. After 3-min (MIP-2) or 10-min (all other stimuli) incubations, the reaction was stopped and cell lysates were prepared in a Triton X-100-based lysis buffer (11, 73), except for immunoprecipitation assays where the lysis buffer was supplemented with 0.1% SDS and 0.5% sodium deoxycholate (RIPA). PLC γ 2 was precipitated using the Q-20 PLC γ 2 antibody and captured using a 1:1 mixture of protein A Sepharose (Invitrogen) and protein G Agarose (Invitrogen). Triton-soluble whole-cell lysates or PLC γ 2 immunoprecipitates were immunoblotted with antibodies against phosphotyrosine (clone 4G10; Millipore), PLC γ 2, p38 MAP kinase (C-20; Santa Cruz Biotechnology, Inc.), ERK1/2 (combination of C-16 [ERK1] and C-14 [ERK2]; Santa Cruz Biotechnology, Inc.), I κ -B α (Cell Signaling Technology), or phosphospecific antibodies (Cell Signaling Technology) against the p38 MAP kinase, ERK, and I κ -B α (14D4).

In vitro and in vivo migration. In vitro migration of neutrophils was assessed by a Transwell assay system essentially as previously described (10, 11). In brief, Transwell inserts with polycarbonate filters of 3- μ m pore size (Corning) were precoated with human fibrinogen, filled with suspensions of WT or PLC γ 2^{-/-} murine neutrophils, and inserted in 24-well plate wells filled with assay media containing varying concentrations of fMLP. The

migration of neutrophils into the lower compartment during a 60-min period was assessed by an acid phosphatase assay as previously described (11).

A competitive migration assay during sterile peritonitis in mixed bone marrow chimeras (11) was used to assess in vivo migration of neutrophils. To this end, bone marrow cells of PLC γ 2^{-/-} mice on the C57BL/6 genetic background (i.e., carrying the CD45.2 allele) were mixed with bone marrow cells from congenic mice expressing CD45.1 on the C57BL/6 genetic background at varying ratios ranging from 10 to 70% of CD45.2-expressing cells. This mixed cell suspension was injected intravenously into lethally irradiated CD45.1-expressing recipient mice, giving rise to mixed bone marrow chimeras carrying CD45.2-expressing PLC γ 2^{-/-} and CD45.1-expressing PLC γ 2^{+/+} hematopoietic cells. To exclude any effect of the different CD45 alleles on cell migration, a few control chimeras were generated in a similar fashion but using PLC γ 2^{+/+} (intact C57BL/6) mice as the CD45.2-expressing donor strain, giving rise to mixed chimeras with CD45.1- and CD45.2-expressing PLC γ 2^{+/+} hematopoietic cells. 5–8 wk after transplantation, the mixed bone marrow chimeras were injected intraperitoneally with 1 ml of 3% thioglycollate broth (Heipha Diagnostics). Blood was taken directly before as well as 2 and 4 h after the injection, and the peritoneal cavity was lavaged at 4 h. The relative percentage of CD45.1- and CD45.2-expressing neutrophils in the peripheral blood and peritoneal lavage samples was determined by flow cytometry in the Gr1-positive granulocyte gate. Relative migration of neutrophils of the CD45.2-positive PLC γ 2^{-/-} or PLC γ 2^{+/+} genotypes (relative to the CD45.1-expressing PLC γ 2^{+/+} cells) was calculated as follows:

$$\text{relative migration} = \frac{\left(\frac{\text{percentage of CD45.2 cells in peritoneum}}{\text{percentage CD45.2 cells in blood}} \right)}{\left(\frac{\text{percentage of CD45.1 cells in peritoneum}}{\text{percentage of CD45.1 cells in blood}} \right)}$$

Intravital microscopy and whole mount cremaster muscle preparation. Bone marrow chimeras were anesthetized using intraperitoneal injection of ketamine and xylazine and the cremaster muscle was prepared for intravital imaging as previously described (63). Intravital microscopy was performed on an upright microscope (BX51; Olympus) with a 40 \times 0.75 NA saline immersion objective. The microcirculation was recorded using a charge-coupled device camera (CF8/1; Kappa) coupled to a recorder (S-VHS; Panasonic). Superfusion of the cremaster muscle and local treatment with 1 μ M fMLP was performed as previously described (63). Postcapillary venules ranged from 25 to 35 μ m in diameter and were observed before and during fMLP administration.

Geometric and hemodynamic parameters, such as vessel diameter, leukocyte diameter, and vessel segment length, of postcapillary venules were assessed from recorded video tapes using a digital image processing system as previously described (77). Spreading of adherent leukocytes in postcapillary venules was assessed by measuring the diameter (height) of attached leukocytes perpendicular to the vessel wall before and at various time points during fMLP superfusion as previously described (63). Mean blood flow velocities and wall shear rates (γ_w) were estimated as previously described (63). Rolling leukocyte flux fraction was defined as the ratio of rolling leukocytes to the total number of leukocytes passing the same vessel per minute (78). Leukocyte adhesion was defined as the number of adherent cells per millimeters squared of vessel surface area (63). Systemic leukocyte concentration was determined from blood samples taken at the end of the experiment as previously described (63).

For whole mount preparations, mouse cremaster muscles were surgically prepared, as described in the first paragraph of this section, and superfused with 1 μ M fMLP in superfusion buffer for 15 min. Thereafter, cremaster muscles were fixed with 4% paraformaldehyde and whole mounts were prepared as previously described (79). Fixed cremaster muscles were stained with Giemsa and analyzed for the number of intravascular and perivascular leukocytes using an upright microscope (Axioskop; Carl Zeiss, Inc.) through a 100 \times 1.3 NA oil immersion objective. Whole mounts from untreated cremaster muscles prepared from both WT and PLC γ 2^{-/-} bone

marrow chimeras served as negative controls. All cremaster muscle experiments were independently assessed by two investigators blinded for the treatment and genotype of the mice.

K/B×N serum transfer arthritis. Mice carrying the KRN T cell receptor transgene (25) on the C57BL/6 genetic background were mated with NOD mice to obtain KRN transgene-positive offsprings on the C57BL/6 × NOD F1 genetic background (K/B×N mice) as well as their transgene-negative (B×N) littermates. The presence of the transgene was determined by flow cytometry as well as by looking for visible signs of arthritis in the K/B×N mice. Blood was taken by retroorbital bleeding and sera from transgene-positive and transgene-negative mice were pooled separately.

Arthritis was induced by intraperitoneal injection of 400 μ l of arthritogenic (K/B×N) or control serum into WT or PLC γ 2^{-/-} bone marrow chimeras or intact (nonchimeric) mice, followed by daily assessment of arthritis development for 2 wk. Visible clinical signs of arthritis were scored on a 0–10 scale by two investigators blinded for the origin and treatment of the mice. Ankle thickness was measured by a spring-loaded caliper (Kroepelin). For histological analysis, mice were killed 4 d after serum transfer and their ankle joints were fixed in formalin (Sigma–Aldrich). The joints were then decalcified, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (Histopathology Llc.). Photomicrographs were taken on a microscope (DMI 6000B; Leica).

To assess articular function, mice were placed on a custom-made wire grid (Charles River Laboratories) with identical wire thickness and spacing to a regular wire cage lid. The wire grid was flipped upside down and the length of time the mice held on to the grid was recorded. This test was performed three times daily during the period of 8–12 d after the serum injection. The obtained data were combined into holding-on curves similar to Kaplan–Meier survival curves.

Online supplemental information. Fig. S1 shows expression level of PLC γ isoforms in neutrophils and provides detailed information about PLC γ antibody specificity. Fig. S2 shows repopulation of PLC γ 2^{-/-} bone marrow chimeras by donor-derived neutrophils after bone marrow transplantation. Fig. S3 shows functional responses of neutrophils from intact (nonchimeric) PLC γ 2^{-/-} mice. Fig. S4 shows CD18-dependent activation of adherent neutrophils by various proinflammatory agonists. Table S1 shows hemodynamic and microvascular parameters in fMLP-stimulated cremaster muscle venules. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20081859/DC1>.

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