ulations of CD45R$^+$ cells in spleens or of CD45R$^+$ and CD43$^+$ cells in bone marrow (*10*). Unlike wild-type mice, however, mice lacking PI3Kγ produced few antibodies containing the λ light chain when immunized with T cell–independent (TI) antigen hydroxylnitrophenyl (NP)–Ficoll (Fig. 4A). By contrast, mice lacking both PLC-β2 and PLC-β3 consistently produced larger amount of TI antigen-specific antibodies composed of the immunoglobulin λ light chain (TI-Igλ$_L$) than did wild-type mice (Fig. 4A). It appears that the PLC pathway, in this case, opposes the PI3K pathway. Enhancement in TI-Igλ$_L$ production appeared to be primarily dependent on the PLC-β3 deficiency (Fig. 4A). Neither PLC nor PI3K deficiency affected the production of TI-Igκ (Fig. 4B) or of T cell–dependent (TD) antigen NP–chicken gamma globulin (NP-CCG)–specific antibodies composed of either λ or κ light chains (*10*). Together these data suggest that the production of TI-Igλ$_L$ may be subjected to regulation by G protein–mediated signaling pathways. Because no differences were detected between wild-type and PI3Kγ-deficient mice in the amount of total serum Igλ$_L$ and in the number of B cells carrying cell surface λ$_L$ (*10*), we think that PI3Kγ deficiency is more likely to affect antigen-dependent processes than early development of B cells.

Mice lacking PLC-β3 developed spontaneous multifocal skin ulcers usually starting at the age of 6 months or older (Fig. 4C). The lesions were localized mainly behind ears or on the neck, but sometimes also appeared on the face. Similar phenotypes were observed with mice lacking both PLC-β2 and PLC-β3. Histological examination of the lesion tissues revealed hyperinfiltration of leukocytes in the lesion tissues (Fig. 4, D and E). Most of the infiltrated leukocytes had morphological characteristics of macrophages and lymphocytes. No ulcerative lesions were observed in wild-type mice, mice heterozygous for the disrupted PLC-β3 genes, or other transgenic lines including PLC-β2– and PI3Kγ-null mice that were housed in the same rooms under the same conditions. This ulcerative phenotype is consistent with the idea that the PLC pathways act to inhibit some important responses mediated by chemoattractants.

In summary, this study with mouse lines deficient in two prominent chemoattractant-activated signaling pathways confirms that both PI3Kγ and PLC-β2/-β3 have important roles in chemoattractant-induced responses. The study also revealed roles for these proteins in leukocyte functions, including the involvement of PI3Kγ in the production of TI-Igλ$_L$ and the PLC pathway in down-modulation of chemotaxis and production of TI-Igλ$_L$ and in hyperinflammatory conditions.

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7. The PI3Kγ-deficient mouse line was generated by standard protocol (*11*). An 8-kb genomic DNA was isolated from a mouse 129sv genomic DNA library containing at least the first three exons of mouse PI3Kγ. A part of the first exon and entire second and third exons were replaced by the cDNA encoding GFP, which was fused in frame with the coding sequence of PI3Kγ. In addition, a neomycin–resistant gene expression unit was inserted behind GFP for selection of transfected embryonic stem (ES) cells. Three of the positive ES clones were used to produce chimeras. Mice heterozygous and homozygous for the disrupted PI3Kγ genes were produced by standard mating schemes.
8. The levels of PtdIns (3,4,5)P_3 were determined as described (*24*) with some modification. Mouse neutrophils (1×10^7) were labeled with [^{32}P]orthophosphate (1 mCi/ml) for 60 min at 37°C. After washing, cells were treated with 1 μM *f*MLP for 45 s. Lipid was extracted and analyzed on a 20 cm by 20 cm Silica Gel 60 thin-layer chromatography plate (EM Science, Gibbstown, NJ), as described in (*24*).
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26. The levels of guanosine triphosphate (GTP)–bound Rac were assayed by determining the amount of Rac associated with glutathione S-transferase–protein binding domain (GST-PBD) as described (*25*), with some modification. Murine neutrophils (1×10^7/50 μl) were treated with 4 μM *f*MLP for the durations indicated. The reaction was stopped by adding the same volume of the 2× lysis buffer [50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 200 mM NaCl, 2% NP-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, leupeptin (2 μg/ml), aprotinin (2 μg/ml), 2 mM orthovanadate, and 10 μg of GST-PBD]. The lysates were centrifuged for 3 min at 700*g*, and the supernatant was incubated with glutathione-Sepharose 4B beads for 1 hour at 4°C after addition of 200 μl of binding buffer [25 mM tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 30 mM $MgCl_2$, 40 mM NaCl, and 0.5% Triton X-100]. The beads were washed three times with a washing buffer (the binding buffer with 1% Triton X-100) and washed one time with the binding buffer in the absence of Triton X-100. The beads were resuspended in SDS–polyacrylamide gel electrophoresis sample buffer and analyzed by Western blot with an antibody to Rac.
27. Mice (8 to 12 weeks old) were injected intraperitoneally with 10 μg of Alumn-precipitated NP32-Ficoll (TI) or NP22-CGG (TD). Sera were collected on day 7. Ten μl of serum was added to 100 μl of phosphate-buffered saline. Enzyme-linked immunosorbent assay (ELISA) was carried out in 96-well plates coated with NP30–bovine serum albumin with an ELISA kit from Zymed (South San Francisco, CA).

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Central Role for G Protein–Coupled Phosphoinositide 3-Kinase γ in Inflammation

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Phosphoinositide 3-kinase (PI3K) activity is crucial for leukocyte function, but the roles of the four receptor-activated isoforms are unclear. Mice lacking heterotrimeric guanine nucleotide-binding protein (G protein)–coupled PI3Kγ were viable and had fully differentiated neutrophils and macrophages. Chemoattractant-stimulated PI3Kγ$^{-/-}$ neutrophils did not produce phosphatidylinositol 3,4,5-trisphosphate, did not activate protein kinase B, and displayed impaired respiratory burst and motility. Peritoneal PI3Kγ-null macrophages showed a reduced migration toward a wide range of chemotactic stimuli and a severely defective accumulation in a septic peritonitis model. These results demonstrate that PI3Kγ is a crucial signaling molecule required for macrophage accumulation in inflammation.

Chemoattractant-mediated recruitment of leukocytes is a key step in the progress of acute and chronic inflammation. Chemokines and chemotactic peptides, such as *N*-formyl-Met-Leu-Phe (*f*MLP), C5a, and interleukin-8 (IL-8), bind to G protein–coupled re-

ceptors (*1*). Receptor activation induces the release of Gβγ subunits from trimeric G proteins. In phagocytic cells, this triggers a series of signaling events that culminate in directional cell movement, phagocytosis, degranulation, and superoxide generation (*1*). The production of phosphatidylinositol 3,4,5-trisphosphate [PtdIns (3,4,5)P_3] appears to have an essential role, because inhibitors of PI3K prevent these responses (*2*). Leukocytes express all the four known class I PI3K isoforms (PI3Kα, β, γ, and δ), but it is presently not clear which enzyme(s) relay inflammatory signals (*3*).

To assess the physiologic role of the PI3Kγ isoform, we generated PI3Kγ-deficient mice by homologous recombination. The targeting vector disrupted the PI3Kγ gene by the insertion of an IRES (internal ribosomal entry site)-LacZ and a neomycin resistance cassette in the first coding exon (exon 2) (*4*). Embryonic stem (ES) cell clones showing heterozygous gene disruption were used to generate germ line chimeras (*5*).

Mice homozygous for the PI3Kγ-targeted allele were viable, fertile, and displayed a normal life-span in a conventional mouse facility. Whereas wild-type (WT) mice expressed PI3Kγ in neutrophils, macrophages, and splenocytes, homozygous mutant cells showed no expression of the protein. In the same cells, the lack of PI3Kγ did not alter the expression of other class I PI3Ks (namely α, β, and δ) (Fig. 1A) (*6*).

Peripheral blood cell counts of PI3Kγ-deficient mice showed no statistically significant differences in the hematocrit or in the distribution of lymphocytes, monocytes, basophils, and eosinophils compared with those in WT animals. By contrast, PI3Kγ-null mice had about twice as many of circulating polymorphonuclear neutrophils (PMNs) as WT mice (Table 1). Microscopic examination of blood smears did not show any morphological abnormality in any leukocyte population.

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Table 1. Blood parameters. Data are the mean ± SE of 15 mice from each group.

Parameter	WT	PI3Kγ$^{-/-}$
Hematocrit (%)	47.30 ± 0.72	47.8 ± 1.03
Neutrophils (10^3/μl)	0.66 ± 0.10	1.23 ± 0.13*
Lymphocytes (10^3/μl)	2.18 ± 0.29	1.26 ± 0.17
Monocytes (10^3/μl)	0.08 ± 0.02	0.08 ± 0.02
Eosinophils (10^3/μl)	0.004 ± 0.001	0.008 ± 0.003
Basophils (10^3/μl)	0.008 ± 0.002	0.006 ± 0.002
Platelets (10^3/μl)	415 ± 25	410 ± 35

*$P < 0.001$ (Student's t test).

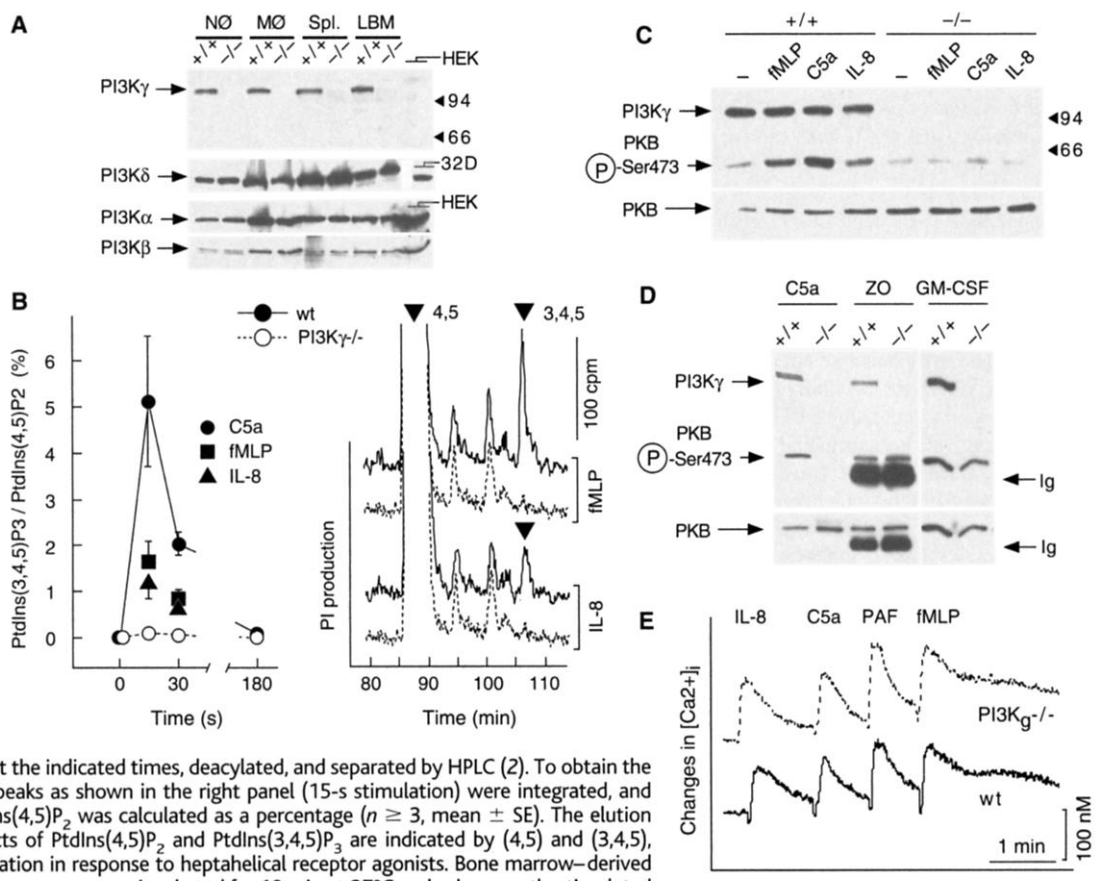

Fig. 1. PI3K downstream signaling by seven–transmembrane helix receptors. (**A**) Failure of PI3Kγ$^{-/-}$ hematopoietic cells to express PI3Kγ. Murine PI3Kγ was detected in WT bone marrow–derived neutrophils (NØ), thioglycollate-elicited peritoneal macrophages (MØ), macrophage-depleted splenocytes (Spl.), and cells from the upper Percoll layer [light bone marrow fraction (*6*), LBM; consisting mainly of lymphocytes and monocytes] by immunoblotting with monoclonal antibodies produced against the NH_2-terminal part of human PI3Kγ. PI3Kα, β, and δ were detected with antibodies described in (*6*). Murine hematopoietic 32D cells (32D) and human embryonic kidney (HEK) 293 cells were applied as positive and negative controls. (**B**) Neutrophil PI3K activity after C5a (10 nM), *f*MLP (1 μM), and IL-8 (100 nM) stimulation. Lipids were extracted from PMNs labeled with inorganic phosphate ($^{32}P_i$) at the indicated times, deacylated, and separated by HPLC (*2*). To obtain the kinetic profile in the left panel, peaks as shown in the right panel (15-s stimulation) were integrated, and the ratio of PtdIns(3,4,5)P_3/PtdIns(4,5)P_2 was calculated as a percentage ($n \geq 3$, mean ± SE). The elution times of the deacylation products of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are indicated by (4,5) and (3,4,5), respectively. (**C**) PKB phosphorylation in response to heptahelical receptor agonists. Bone marrow–derived PMNs (10^6/ml) of the indicated genotypes were incubated for 10 min at 37°C and subsequently stimulated with 1 μM *f*MLP, 10 nM C5a, or 10 nM IL-8 for 30 s. Samples were probed for the presence of PI3Kγ, PKB phosphorylated on Ser-473, and total PKB. (**D**) PKB phosphorylation by G protein–independent signaling pathways. Human serum–opsonized zymosan (C3b$_i$ and immunoglobulin G–coated) and GM-CSF (100 ng/ml) were used to stimulate neutrophils for 15 and 5 min, respectively. C5a stimulation was done as in (C). (**E**) G protein–dependent intracellular calcium release. Fura-2–loaded PMNs obtained from wild-type (wt) and PI3Kγ-null mice were stimulated with IL-8 (50 nM), C5a (1 nM), platelet activating factor (PAF, 100 nM), and *f*MLP (100 nM).

Fluorescence-activated cell sorter (FACS) analysis of bone marrow PMNs and resident peritoneal macrophages with antibodies to distinct cell surface markers (Gr-1 and CD11b for PMNs; CD11b, F4/80, CD80, and CD86 for macrophages) revealed matching cell distribution and expression patterns in WT and PI3Kγ-null mice, indicating that differentiation of myeloid cells is independent of PI3Kγ (*7*).

The coupling of PI3Kγ to seven–transmembrane receptor signaling was assessed in morphologically mature bone marrow PMNs. Phosphoinositides were analyzed by metabolic labeling with ^{32}P–inorganic phosphate and subsequent analysis of deacylated lipids on high-performance liquid chromatography (HPLC). After stimulation of cells with C5a, *f*MLP, or IL-8, PtdIns(3,4,5)P_3 was produced in WT but not in PI3Kγ$^{-/-}$ cells (Fig. 1B). The serine-threonine protein kinase B (PKB/Akt) is a major target of PI3K (*8*). Whereas C5a, fMLP, and IL-8 triggered PKB phosphorylation in response to chemoattractants in WT cells, in PI3Kγ-null PMNs PKB phosphorylation did not rise above background levels (Fig. 1C). Serum–opsonized zymosan and granulocyte-macrophage colony-stimulating factor (GM-CSF), by contrast, were still capable of signaling to PKB (Fig. 1D). Therefore, protein tyrosine kinase–dependent processes successfully activate PI3Kα, β, or δ isoforms in PI3Kγ-null cells. The intact calcium release initiated by *f*MLP-, C5a-, platelet-activating factor (PAF), and IL-8 receptors (Fig. 1E) illustrates that PI3Kγ-independent G protein–coupled signaling pathways are not affected. PI3Kγ is thus the sole PI3K isoform coupled to *f*MLP, C5a, and IL-8 receptors.

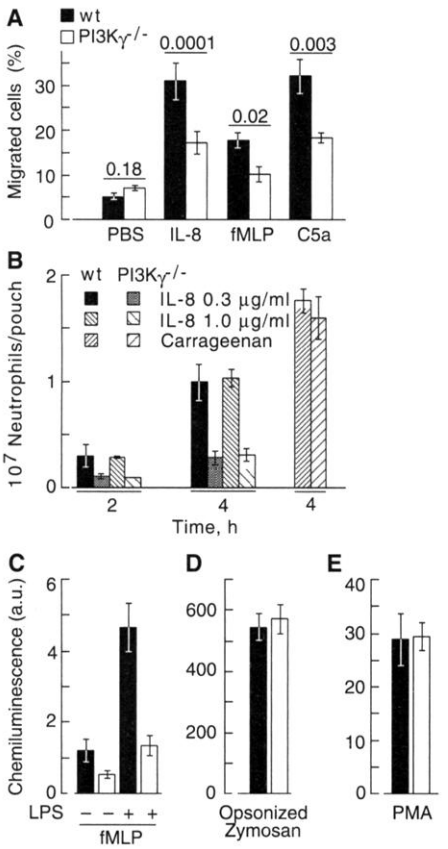

Fig. 2. Chemotaxis and respiratory burst of PMNs. (**A**) Chemotaxis to IL-8 (50 nM), *f*MLP (100 nM), and C5a (3 nM) was measured with fluorescently labeled cells (*18*). The data (mean ± SE, n = 6 to 13) are expressed as the percentage of total cells that migrated. Indicated P values were calculated by ANOVA and represent comparisons of WT and PI3Kγ-null populations. (**B**) Migration to air pouches. IL-8 (1 and 0.3 μg/ml in sterile apyrogenic saline) and carrageenan (1% in sterile apyrogenic saline) were injected into 6-day-old air pouches (*12*). Mice were killed 2 or 4 hours later and the exudate collected in 1 ml of saline. Results are the mean ± SE of PMN counts from five to six different mice per group. (**C** to **E**) Respiratory burst in PMNs. (C) PMNs were preincubated in the absence or presence of lipopolysaccharide (LPS, 100 ng/ml) before stimulation with 1 μM *f*MLP (mean ± SE; n = 8 to 13). (D) Resting PMNs were stimulated with human serum–opsonized zymosan (mean ± SE; n = 3). (E) Resting PMNs were stimulated with 100 nM PMA (mean ± SE; n = 6). Chemiluminescence was measured according to published methods (*19*), and data represent integrated responses (*f*MLP, 3 min; opsonized zymosan, 30 min; PMA, 30 min).

Of the various neutrophil responses, the agonist-induced respiratory burst is the most sensitive to PI3K inhibitors (*2*). Resting neutrophils only weakly respond with a respiratory burst to seven–transmembrane receptor agonists, but can be "primed" with tumor necrosis factor–α, GM-CSF, or lipopolysaccharide (LPS) to markedly increase their response (*13*). Consistent with these data, WT murine bone marrow–derived PMNs responded to *f*MLP after a prolonged incubation with LPS, whereas PI3Kγ-null cells remained less responsive (Fig. 2C). Prolonged adhesion restored the sensitivity of PI3Kγ-null neutrophils to *f*MLP in a wortmannin-sensitive manner (*7*). Activation of the NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidase by serum–opsonized zymosan or phorbol 12-myristate 13-acetate (PMA) was intact in PI3Kγ$^{-/-}$ cells (Fig. 2, D and E). These results, and the reported sensitivity of *f*MLP and zymosan-induced respiratory burst to wortmannin (*2*), suggest that *f*MLP triggers PtdIns(3,4,5)P_3 production required for the respiratory burst exclusively by way of PI3Kγ. The zymosan signal (complement 3b_i-mediated stimulation) or priming events, on the other hand, act by way of protein tyrosine kinases on p85-associated PI3Ks, thus relieving an essential requirement for PI3Kγ (e.g., for the activation of PKB; see Fig. 1D).

PI3Kγ functions in cytoskeletal remodeling and leukocyte mobility (*9*). In addition, the chemotactic response to agonists of G protein–linked heptahelical receptors is correlated with PI3K-dependent activation of PKB (*10*). We thus examined the ability of mutant PMNs to adhere and migrate. Mutant cells showed no increase in cell adhesion on fibronectin in response to IL-8 (*11*). Because adhesion and cytoskeletal remodeling are prerequisites for cell motility, the chemotactic response to IL-8, *f*MLP, and C5a was assayed. PMNs from PI3Kγ-null mice displayed a reduction in chemotaxis in response to IL-8, *f*MLP, and C5a in vitro (Fig. 2A). The in vivo impact of this chemotactic defect was assessed by measurement of agonist-induced PMN infiltration into subcutaneous air pouches. IL-8 at doses of 0.3 and 1 μg caused recruitment of 60% fewer PMNs in PI3Kγ-null mice than in WT animals after 2 and 4 hours (Fig. 2B). The response to carrageenan, a pleiotropic inflammatory stimulus (*12*), was not altered in PI3Kγ-null mice (Fig. 2B). These data indicate that lack of PI3Kγ leads to impaired recruitment of PMNs in response to chemokines.

PI3Kγ-null macrophages, obtained from peritoneal exudate of thioglycollate-treated mice, were tested in an in vitro chemotaxis assay with various chemoattractants. First, we evaluated the chemotactic response toward endotoxin-activated mouse serum (EAMS) as a source of chemotactic complement fractions. In this assay, the chemotactic response of PI3Kγ-null cells was reduced by 60% (Fig. 3A). In contrast, peritoneal macrophages showed a similar migration toward PMA, indicating that the defect resided in receptor signaling rather than in leukocyte locomotion ability. To further characterize the migratory deficiency observed with EAMS, we stimulated PI3Kγ-deficient macrophages with G protein–coupled serpentine receptor agonists such as RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein–5 (MIP-5), macrophage-derived chemokine (MDC), stromal cell–derived factor–1 (SDF-1), and C5a. Migration toward all of these chemotactic agents was reduced in mutant macrophages. Chemotaxis of PI3Kγ$^{-/-}$ macrophages was decreased in response to C5a (85% reduction), SDF-1 (85%), RANTES (80%), MDC (70%), and MIP-5 (52%) compared with WT cells (Fig. 3A). PI3Kγ$^{-/-}$ peritoneal macrophages also exhibited an 85% reduction in chemotaxis toward vascular endothelial growth factor (VEGF), an agonist known to bind a tyrosine kinase receptor (Fig. 3A) (*14*). This observation is consistent with the report that VEGF-stimulated migration can be inhibited by pertussis toxin (*15*) and establishes a crucial role of PI3Kγ in this G protein–mediated response. Upon stimulation with G protein–coupled receptor agonists RANTES, MIP-5, SDF-1, C5a, and *f*MLP, mutant and WT macrophages showed similar increases in intracellular Ca^{2+} release (Fig. 3B). Consistent with a specific PI3K signaling defect, PI3Kγ$^{-/-}$-purified resting peritoneal macrophages showed no increase in PKB phosphorylation after C5a stimulation (Fig. 3C).

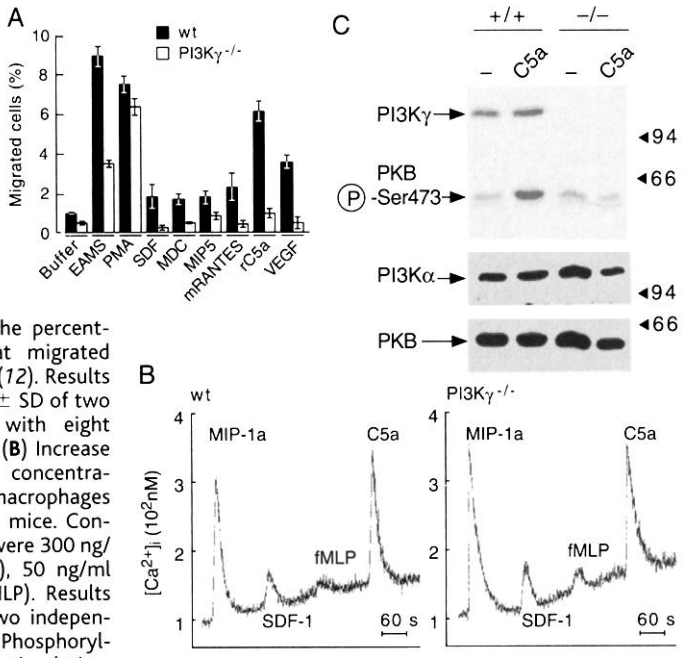

Fig. 3. Role of PI3Kγ in thioglycollate-elicited peritoneal macrophages. (**A**) Chemotaxis was elicited by using optimal concentrations of agonists: 5% EAMS, 16 nM PMA, 100 ng/ml SDF-1, 100 ng/ml MDC, 1 μg/ml MIP-5, 100 ng/ml RANTES, 100 ng/ml C5a, and 10 ng/ml VEGF. Data represent the percentage of total cells that migrated through the filter pores (*12*). Results are shown as the mean ± SD of two triplicate experiments with eight mice of each genotype. (**B**) Increase of intracellular calcium concentration in Fura-2–loaded macrophages from WT and PI3Kγ$^{-/-}$ mice. Concentrations of agonists were 300 ng/ml (MIP-1α and SDF-1), 50 ng/ml (C5a), and 10 μM (*f*MLP). Results are representative of two independent experiments. (**C**) Phosphorylation of PKB after C5a stimulation. Experiments were performed as in Fig. 1C.

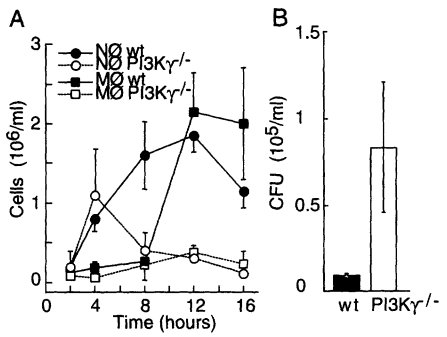

Fig. 4. Impairment of leukocyte recruitment during septic peritonitis in PI3Kγ$^{-/-}$ mice. (**A**) Kinetics of PMN and macrophage recruitment after intraperitoneal administration of 10^7 CFU of *E. coli* [American Type Culture Collection (ATCC) 25922]. Bacteria were grown to exponential phase, and the optical density at 600 nm was used to extrapolate cell number. Cells were washed and resuspended in 200 μl of phosphate-buffered saline before injection. Three to six mice were used for each time point (mean ± SD). (**B**) Clearance of viable *S. aureus* (ATCC 25923) from the peritoneal cavity. The peritoneal cavity was washed with 5 ml of sterile saline and the number of CFU/ml was evaluated. Results represent the mean ± SD of three mice of each genotype.

A model of aseptic peritonitis induced by intraperitoneal injection of thioglycollate was used to evaluate the impact of the lack of PI3Kγ on the onset of an inflammatory response in vivo. The number of thioglycollate-elicited peritoneal leukocytes was measured at various time points in mutant and control mice. There was a 36% decrease in total PI3Kγ$^{-/-}$ peritoneal leukocytes ($n = 7$; $P = 0.09$) at 120 hours, but no differences were present at earlier time points (4 and 48 hours). In contrast, induction of septic peritonitis by injection of Gram-positive and Gram-negative bacteria resulted in an impaired inflammatory response in PI3Kγ-deficient mice. FACS analysis and microscopic inspection of the elicited cell populations indicated that the lack of PI3Kγ affected the recruitment of both neutrophils and macrophages. The number of peritoneal PI3Kγ-null macrophages as early as 12 hours after bacteria administration was reduced by 90% compared with that in WT animals (Fig. 4A). Similar results were obtained with Gram-positive bacteria such as *Staphylococcus aureus* [5×10^8 colony-forming units (CFU)]. Microscopic analysis of peritoneal leukocytes revealed that PI3Kγ$^{-/-}$ macrophages did normally phagocytose bacteria. Because macrophage recruitment is essential to purge peritoneal infections, we tested whether PI3Kγ-deficient mice were able to clear peritoneal bacteria after administration of sublethal doses of *S. aureus* (*16*). Forty-eight hours after intraperitoneal injection of 5×10^7 CFU per mouse, bacteria persisted in the abdominal cavity of PI3Kγ$^{-/-}$ mice, with a concentration 10 times that of WT mice (Fig. 4B).

Our data are consistent with a central role of PI3Kγ in linking G protein–coupled receptor signaling to PtdIns(3,4,5)P_3 production, which in turn rigorously governs cell motility in macrophages and to some extent in neutrophils. The control of macrophage infiltration in chronic inflammatory diseases such as rheumatoid arthritis, pulmonary fibrosis, atherosclerosis, and autoimmune disorders is a major task of present pharmacological research. Our results indicate that PI3Kγ might be a suitable target for development of drugs that could specifically modulate phagocyte functions without generating severe side effects.

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4. A genomic DNA fragment of 11 kb encompassing exons 1 through 8 of the mouse PI3Kγ was used to integrate the IRES-LacZ cassette followed by the PGK-neomycin resistance gene from the pWH9 plasmid (provided by R. Fässler, Lund University, Sweden) in exon 2, 105 base pairs downstream of the first coding ATG. Five independently targeted R1 ES cell clones were identified by Southern blot hybridization. No evidence for random integration was detected (*17*).
5. All ES cells clones were injected into C57BL/6 blastocysts. For genotyping of mice, DNA derived from tail biopsies was amplified by polymerase chain reaction with two primers sets (1: 5′-GGAGAACTATGAACAACCGG-3′, 5′-CAACTTCCAGTAATGCAGGC-3′; 2: 5′-CTGCTCTTTACTGAAGGCTC-3′, 5′-CAACTTCCAGTAATGCAGGC-3′) that detect the WT and targeted allele, respectively. Phenotypic analysis was performed on two lines derived from independent clones, and results were confirmed in a 129sv-C57BL/6 mixed and 129sv inbred genetic background. The IRES-LacZ reporter gene under the control of the PI3Kγ promoter was expressed in peripheral blood leukocytes and in spleen macrophages [(*17*); for expression studies see H. G. Bernstein, G. Keilhoff, M. Reiser, S. Freese, R. Wetzker, *Cell Mol. Biol.* **6**, 973 (1998)]. Animal experiments were carried out in accordance with institutional guidelines.
6. Bone marrow cells were flushed from femurs with ice-cold RPMI 1640 medium (Gibco/BRL) supplemented with 10% fetal calf serum and antibiotics. Erythrocytes were removed by isotonic lysis, and white blood cells were loaded onto a Percoll 60/80% discontinuous step gradient. PMNs were collected from the 60/80% interface after a 30-min centrifugation (4°C, 520*g*). Splenocytes were depleted of macrophages (3-hour incubation at 37°C, 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM medium, Life Technologies). Macrophages were obtained by washing the peritoneal cavity of mice 5 days after intraperitoneal injection of 1 ml of 3% thioglycollate (Difco). Antibodies to PI3K were from A. Klippel (Berlin, Germany; PI3Kα), R. Wetzker (Jena, Germany; PI3Kγ), B. Vanhaesebroeck (London, UK; PI3Kδ), and St. Cruz Biotechnology (PI3Kβ).
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adhesion were not significantly reduced by the loss of PI3Kγ. Teflon-coated 12-well glass slides (Marienfeld) were coated with fibronectin (20 μg/ml; Sigma) solution. Calcein-AM (Molecular Probes)–loaded PMNs (20 μl) were applied to the glass slides. After stimulation, nonadherent cells were removed by washing. Fluorescence of attached cells was measured in a Bio-Tek FL600 fluorescence plate reader (excitation, 485 nm, 20-nm slit; emission, 530 nm, 25-nm slit).
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Requirement for DARPP-32 in Progesterone-Facilitated Sexual Receptivity in Female Rats and Mice

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DARPP-32, a dopamine- and adenosine 3′,5′-monophosphate (cAMP)–regulated phosphoprotein (32 kilodaltons in size), is an obligate intermediate in progesterone (P)–facilitated sexual receptivity in female rats and mice. The facilitative effect of P on sexual receptivity in female rats was blocked by antisense oligonucleotides to DARPP-32. Homozygous mice carrying a null mutation for the DARPP-32 gene exhibited minimal levels of P-facilitated sexual receptivity when compared to their wild-type littermates. P significantly increased hypothalamic cAMP levels and cAMP-dependent protein kinase activity. These increases were not inhibited by a D_1 subclass dopamine receptor antagonist. P also enhanced phosphorylation of DARPP-32 on threonine 34 in the hypothalamus of mice. DARPP-32 activation is thus an obligatory step in progestin receptor regulation of sexual receptivity in rats and mice.

Progesterone (P) and dopamine (DA) facilitation of sexual receptivity in female rats requires intact, intracellular progestin receptors (PRs) (*1*). Wild-type female mice exhibit high levels of P- and DA-facilitated lordosis, whereas homozygous females carrying a null mutation for the PR gene show minimal reproductive behavior (*2*, *3*). These observations substantiate a critical role for the PR as a transcriptional mediator for the signal transduction pathways initiated by P and DA.

DA, signaling through the D_1 subclass of receptors in the neostriatum, induces increases in the levels of adenosine 3′,5′-monophosphate (cAMP) and activates cAMP-dependent protein kinase (PKA) (*4*). Dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32) is phosphorylated by PKA. In its phosphorylated state, this molecule, by inhibiting the activity of protein phosphatase–1 (PP-1), increases the state of phosphorylation of many substrate proteins, leading to the induction of physiological responses (*4*). To determine whether DARPP-32 might be involved in P and DA actions on the hypothalamus, we examined its role in the facilitation of sexual receptivity in female rats and mice (*5*).

Antisense oligonucleotides to the PR inhibit P-facilitated lordosis in female rats (*6*, *7*). We used a similar strategy to examine the role of DARPP-32 in P- and DA-facilitated sexual receptivity. Ovariectomized, estradiol benzoate (EB)–primed, Sprague-Dawley female rats with stereotaxically implanted stainless steel cannulae in the third cerebral ventricle (*5*) exhibited high levels of P-facilitated lordosis in the presence of males (Fig. 1A). This P-facilitated lordosis response was significantly reduced in the animals that received antisense oligonucleotides to DARPP-32 but not in control animals receiving sense oligonucleotides to DARPP-32 (Fig. 1A).

In a parallel experiment, intracerebroventricular (icv) administration of the selective D_1 agonist SKF 38393 also facilitated a lordosis response in EB-primed rats. The response was reduced by antisense but not by sense oligonucleotides to DARPP-32 (Fig. 1A). In contrast, antisense oligonucleotides to DARPP-32 had no effect on serotonin-facilitated sexual receptivity in these animals (Fig. 1B). These results were confirmed with two separate sets of oligonucleotides to DARPP-32 mRNA and their matched sense oligonucleotide controls.

DA and P facilitation of sexual receptivity were also examined in mice carrying a null mutation for the gene encoding DARPP-32 (*8*). Wild-type and DARPP-32 knockout mice show similar levels of hypothalamic PRs (*9*). Ovariectomized wild-type, heterozygous, and homozygous female mice were tested for a lordosis response in the presence of wild-type DARPP-32 males 30 min after P administration (*3*, *5*). Icv P after EB priming resulted in high levels of lordosis in wild-type and heterozygous mice, whereas homozygous mice exhibited significantly lower levels of lordosis (Fig. 2A). The lordosis response of the wild-type mice to the treatments did not differ from those of the parental mouse strains C57BL/6 and 129SvEv, indicating that the behavioral alterations observed in knockout mice were not due to variations in genetic background.

Icv administration of SKF 38393 48 hours after EB priming also facilitated a reliable lordosis response in the parental strains and in wild-type and heterozygous female mice. Homozygous mutant mice, however, responded to the icv injection of SKF 38393 with minimal levels of lordosis (Fig. 2B). The lordosis response did not significantly differ between wild-type, heterozygous, and homozygous mice upon icv injection of serotonin (Fig. 2B), corroborating the DARPP-32 antisense experiments in rats indicating that DARPP-32 is not an integral part of the serotonin signaling pathway. This is consis-

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