Phosphoinositide 3-Kinases γ and δ, Linkers of Coordinate C5a Receptor-Fcγ Receptor Activation and Immune Complex-induced Inflammation*

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Stephanie Konrad1, Syed R. Ali1,2, Kristina Wiege3, Shahzad N. Syed1, Linda Engling1, Roland P. Piekorz1, Emilio Hirsch1, Bernd Nürnberg1, Reinhold E. Schmidt1, and J. Engelbert Gessner1,3

From the 1Molecular Immunology Research Unit, Clinic for Immunology and Rheumatology, and the 3SFB587 Collaborative Research Program of the Deutsche Forschungsgemeinschaft, Hanover Medical School, 30625 Hanover, Germany, the 2Department of Biochemistry and Molecular Biology II, Heinrich-Heine University, 40225 Düsseldorf, Germany, and the 1Department of Genetics, Biology, and Biochemistry, University of Torino, 10124 Torino, Italy

Fcy receptors (FcyR) and the C5a receptor (C5aR) are key effectors of the acute inflammatory response to IgG immune complexes (IC). Their coordinated activation is critical in IC-induced diseases, although the significance of combined signaling by these two different receptor classes in tissue injury is unclear. Here we used the mouse model of the passive reverse lung Arthus reaction to define their requirements for distinct phosphoinositide 3-kinase (PI3K) activities in vivo. We show that genetic deletion of class IB PI3Kγ abrogates C5aR signaling that is crucial for FcyR-mediated activation of lung macrophages. Thus, in PI3Kγ−/− mice, IgG IC-induced FcγR regulation, cytokine release, and neutrophil recruitment were blunted. Notably, however, C5a production occurred normally in PI3Kγ−/− mice but was impaired in PI3Kδ−/− mice. Consequently, class IA PI3Kδ deficiency caused resistance to acute IC lung injury. These results demonstrate that PI3Kγ and PI3Kδ coordinate the inflammatory effects of C5aR and FcγR and define PI3Kδ as a novel and essential element of FcγR signaling in the generation of C5a in IC disease.

Activated complement component C5a is a pleiotropic molecule that regulates the activity of many cell types, with a broad range of biological functions in the immune system (1). C5a binds to at least two seven-transmembrane domain receptors, C5aR4 (CD88) and C5L2, expressed on a variety of immune cells, including circulating leukocytes, mast cells, basophils, macrophages, and many others. C5aR-dependent activation of these cells by C5a results in inflammatory mediator release and granule secretion, which in turn alters vascular permeability, induces smooth muscle contraction, and promotes cell migration (2). It is well established that this C5a-triggered cascade of events contributes to the pathogenesis of various diseases in humans, including myocardial ischemia/reperfusion injury and respiratory distress syndrome (3–5). In addition, genetic deletion of C5aR is very effective in preventing inflammation in animal models of type III hypersensitivity (as modeled by the passive reverse Arthus reaction) and arthritis, as well as antibody-dependent type II autoimmunity (6–8).

Complement activation occurs through multiple pathways (classical, alternative, and lectin binding) in the circulation, each of which produces C5a. Interestingly, C5a is also formed within the extravascular tissue compartments through activation of resident innate immune effector cells, such as tissue macrophages, and requires the presence of receptors for the Fc portion of IgG, FcγR (reviewed in Ref. 9). FcγR exert their function through paired expression of activating (FcyRI, FcγRIII, and FcγRIV) and inhibitory (FcyRIIB) receptors (reviewed in Ref. 10). Compelling evidence suggests that the ratio of the opposing signaling FcγR is critical in setting the cellular thresholds for the pathogenic activity of autoantibodies (reviewed in Refs. 10–12) and that C5a regulates this ratio, thus amplifying the FcγR-mediated inflammatory response in autoimmunity (8, 13–15).

Although C5a/C5aR and FcγR likely cooperate in the context of immunological diseases, the precise molecular mechanisms of their combined signaling remain to be elucidated. C5a/C5aR has recently been shown to induce and suppress transcription of the two FcγRIII and FcγRII genes on macrophages in a PI3K-dependent manner (16). In addition, PI3K-mediated signal transduction is also known for FcγR-induced cell activation (17). Distinct members of the PI3K family of signaling molecules may thus participate in the regulatory cross-talk between individual FcγR and C5aR, which was previously proposed to be

inflammatory protein; KC, cytokine-induced neutrophil chemoattractant; TNFα, tumor necrosis factor-α; RT, reverse transcription; AM, alveolar macrophage; mAb, monoclonal Ab.

1 Both authors contributed equally to this work.
2 Recipient of a fellowship of the international M.D./Ph.D. program provided by the Freundesgesellschaft der Medizinische Hochschule Hannover. Present address: Dept. of Pharmacology, University of California, San Diego, La Jolla, CA 92039-0636.
3 To whom correspondence should be addressed: Clinic for Immunology and Rheumatology, Hanover Medical School, Carl-Neuberg-Str. 1, 30625 Hanover, Germany. Fax: 49-511-532-5648; E-mail: gessner.johannes@mh-hannover.de.
4 The abbreviations used are: C5aR, C5a receptor(s); FcγR, Fcγ receptor(s); PI3K, phosphoinositide 3-kinase; IC, immune complex(es); WT, wild-type; OVA, ovalbumin; Ab, antibody; Ag, antigen; BAL, bronchoalveolar lavage; BALF, BAL fluid; PMN, polymorphonuclear leukocyte(s); MIP, macrophage...
a key event in the initiation of the inflammatory cascade in vivo (8, 9, 13, 14).

The PI3K family can be divided into three subfamilies (I, II, and III) on the basis of their structural characteristics, activation mechanisms, and substrate specificity. Class IA PI3K members are heterodimers consisting of a regulatory subunit (seven distinct isoforms including splice variants are known, which are derived from five genes: p85α, p85β, p55α, p55β, and p55γ) and a catalytic subunit (which is one of three types: p110α, p110β, and p110δ) (reviewed in Refs. 18–21). Whereas class IA PI3Kα and PI3Kβ are widely expressed, PI3Kδ is found primarily in cells of the immune system, where it is activated by receptors involved in protein-tyrosine kinase signaling, such as cytokine receptors, antigen receptors present on T and B cells, and the mast cell IgE Fc receptor, FceRI (reviewed in Refs. 22–24). The only class IB PI3K enzyme, PI3Kγ, also consists of a catalytic (i.e. p110γ) and a regulatory (i.e. p101 or p87) subunit and is also preferentially expressed in immune cells (reviewed in Ref. 25). In contrast to PI3Kδ, however, class IB PI3Kγ is predominantly activated by G-protein-coupled receptors, such as C5aR.

Recent studies in mice lacking PI3Kγ and PI3Kδ (26–29) have revealed a key role of class I PI3K members in many cellular immune responses at the interface of innate and adaptive immunity. For instance, PI3Kγ deficiency leads to defects in chemokine-induced migration of neutrophils, macrophages, and dendritic cells to sites of infection and tissue injury (27–30). Moreover, G-protein-coupled receptor-induced activation of mast cells and platelets is also affected in PI3Kδ mice (31, 32), whereas PI3Kδ−/− mice show a severely impaired B cell response of antibody production to T cell-dependent and -independent antigens (26, 33–36).

In this study, we analyzed PI3Kγ−/− mice in the experimental model of the pulmonary Arthus reaction and found defective C5aR-mediated FcγR regulation and macrophage activation, leading to impaired induction of the inflammatory response. Notably, however, C5a production was not affected in PI3Kγ−/− mice, but was impaired in the genetic absence of PI3Kδ. Conse-
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**EXPERIMENTAL PROCEDURES**

**Mice**

The generation of B6 PI3Kγ- and PI3Kδ-deficient mice and their phenotypic characterization have been described previously in detail (26, 27). WT B6 mice were used for all comparisons. All mice were maintained under dry barrier conditions at the animal facilities of the Hanover Medical School and Heinrich-Heine University.

**Passive Reverse Lung Arthus Reaction**

Mice were anesthetized with ketamine and xylazine; the trachea was cannulated; and 150 μg of protein G-purified anti-OVA IgG Ab (ICN) was applied. Immediately thereafter, 20 mg/kg OVA Ag was given intravenously. Ab control animals received phosphate-buffered saline instead of OVA Ag. In PI3K-blocking experiments, 2 μg of wortmannin (Calbiochem) was given intratracheally together with the application of anti-OVA IgG. Mice were killed at 2–4 h after initiation of lung inflammation. BAL was performed five times with 1 ml of 0.9% NaCl at 4 °C. The total cell count of BALF was assessed with a hemocytometer. The amount of red blood cells represented the degree of hemorrhage. For quantitation of PMN accumulation, differential cell counts were performed on Cytospins (10 min at 55 × g) stained with May-Grünwald/Giemsa using 300–500 μl of BALF. The concentrations of MIP-1α, MIP-2, KC, and TNFα in BALF were measured in duplicate in appropriately diluted samples with enzyme-linked immunosorbent assay kits (R&D Systems) according to the manufacturer’s instructions.

**FcγR Expression Analysis in Vitro and in Vivo**

Luciferase Reporter Gene Assays—The FcγR promoter-reporter plasmids FcγRIII p(−808/+18)Luc (0.7 μg) were cotransfected with 0.3 μg of the reference plasmid pRL-CMV into 5 × 10⁵ macrophage MH-S cells in 12-well plates using Lipofectamine™ as

FIGURE 2. **PI3Kγ-dependent FcγR regulation in vitro and in vivo.** A, shown are the relative promoter activities after 48 h of transfection of FcγRII (p−808/+18)Luc (left panel) and FcγRII (p−729/+501)Luc (right panel) into MH-S cells stimulated for 4 h with C5a and treated with the indicated cell-permeable inhibitors (PP2, wortmannin, enethiazolidine-2,4-dione, a selective inhibitor of class IB PI3Kγ) or a neutralizing anti-C5aR mAb. Results represent the means ± S.E. of three independent transfections performed in duplicate. Differences between native (medium) and C5a-induced (C5a) levels of FcγR promoter activities are significant (*, p < 0.05). Differences in C5a compared with C5a + wortmannin, C5a + PI3Kγ inhibitor, and C5a + anti-C5aR antibody treatment groups are significant (*, p < 0.05). B, BAL-AM cells were isolated from WT and PI3Kγ−/− mice at 0 h (gray bars) and 2 h (black bars) after intratracheal instillation of recombinant human C5a. Real-time RT-PCR analysis revealed increased FcγRILuc (left panel) and FcγRILuc (middle panel) and reduced FcγRII (right panel) mRNAs in WT mice but not in BAL-AM cells of PI3Kγ−/− mice following recombinant human C5a treatment. Results are shown as the means ± S.E. (n = three to four mice for each group). Significant differences were determined by Student’s t test (*, p < 0.05). AU, arbitrary units. C, flow cytometric analysis was performed with BAL-AM cells obtained from 0-h (gray bars) and 4-h (black bars) IC-challenged WT and PI3Kγ−/− mice. Both representative (left panels) and quantitative (right panels) results are shown. The quantitative results are expressed as the x-fold change in mean fluorescence intensity (MFI) ± S.E. Differences in 0-h IC compared with 4-h IC treatment groups are significant (*, p < 0.05).

quently, PI3Kδ−/− mice failed also to develop IC-induced inflammation, indicating that both PI3Kγ and PI3Kδ are critical linkers of coordinate C5aR-FcγR activation in IC disease.
Cells were recovered after 24 h, cultured for 24 h in 1% fetal calf serum-containing RPMI 1640 medium, and further treated with 50 ng of recombinant human C5a for 4 h. In some experiments, cells were pretreated for 1 h with an anti-C5aR Ab to block C5aR activity. In PI3K inhibition experiments, wortmannin (20 \( \mu \)M) and a PI3K-specific inhibitor (5-quinoxalin-6-ylmethylenethiazolidine-2,4-dione, 10 nM; Calbiochem) were used. The Src kinase inhibitor PP2, which does not inhibit C5aR signaling (16), served as a negative control. Cells were then lysed and measured for luciferase activities using the Dual-Luciferase reporter assay system (Promega). Firefly luciferase activity was normalized by Renilla luciferase activity to yield the relative promoter activity.

**FIGURE 3. Impaired IC inflammation in PI3K\(\gamma\)-deficient B6 mice.** WT B6 and PI3K\(\gamma\)^−/− mice received 150 \( \mu \)g of anti-OVA Ab intratracheally and 20 mg/kg OVA Ag intravenously, and the inflammatory response in the lung was allowed to proceed for 4 h (IC). Mice not receiving OVA Ag served as Ab controls (Ab). Lungs were lavaged. PMN influx in the alveolar space (A); hemorrhage (B); chemotactic activity (C); and production of MIP-2 (D), MIP-1\(\alpha\) (E), and TNF\(\alpha\) (F) were evaluated. The results are expressed as the means ± S.E. (n = four to nine mice for each group). Differences in IC treatment groups of WT mice compared with PI3K\(\gamma\)^−/− mice are significant or highly significant for all parameters (*, \( p < 0.05 \); **, \( p < 0.001 \)). RBC, red blood cells.

**C5/C5a Analysis**

**Real-time RT-PCR**—Total RNA was prepared from BAL-AM cells of mice at 2 h after Ab and IC treatments and analyzed for C5 transcription by real-time RT-PCR as described (8).

**Detection of C5a-dependent Chemotactic Activity in Vivo**—Bone marrow cells (containing 64–68% PMN) from C5aR-deficient mice or B6 controls were suspended at 7.5 \( \times \) 10^5 cells/ml of RPMI 1640 medium and 0.1% bovine serum albumin (fatty acid-free). 100 \( \mu \)l of the bone marrow cell suspension was placed into the insert of a Transwell chemotaxis chamber, and the bottom well was filled with 600 \( \mu \)l of RPMI 1640 medium and 0.1% bovine serum albumin (negative control) or BALF diluted 1:2 in RPMI 1640 medium and 0.1% bovine serum albumin. BALF was obtained from PI3K\(\gamma\)^−/− and PI3K\(\delta\)^−/− mice, wortmannin-treated B6 mice, or WT controls at 2–4 h after...
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OVA/anti-OVA IC inflammation. BALF from mice receiving Ab but not OVA Ag served as controls. Inserts were combined in the lower chambers and incubated at 37 °C and 6% CO₂ for 2 h. After the incubation, 50 μl of 70 mM EDTA was added to the lower chambers to release adherent cells from the lower surface of the membrane and from the bottom of the well. Plates were further incubated for 30 min at 4 °C; inserts were removed; and the transmigrated neutrophils were vigorously suspended and counted with a FACS Calibur for 1 min at 12 μl/min with gating on forward and side scatter. Migration of PMN from the insert to the bottom well was quantitated as the percentage of total PMN loaded into the upper chamber.

Statistical Analysis

Data for comparison of mean values among samples were analyzed by a two-sided unpaired Student’s t test.

RESULTS

In Vivo Effects of General PI3K Inhibition in IgG IC-induced Inflammation—To determine whether inhibition of PI3K signaling has a protective effect on IgG IC inflammation, we conducted lung Arthus experiments in mice receiving wortmannin, a general PI3K inhibitor. Assessment of lung inflammation by histology demonstrated lung Arthus experiments in mice receiving wortmannin, a general PI3K inhibitor. Assessment of lung inflammation conducted lung Arthus experiments in mice receiving wortmannin, a general PI3K inhibitor. Assessment of lung inflammation by histology revealed that the lungs of mice receiving wortmannin were more inflamed than those of control mice (Fig. 1, A–C). In addition, PI3K inhibition resulted in strong reduction of IC-induced mediator production of CXC chemokines (MIP-2 and KC), MIP-1α, and TNFα (p < 0.001) (Fig. 1, D–F). These data indicate that PI3K activity is required for the generation of crucial inflammatory mediators in the initiation of acute IC inflammation, thus confirming previous observations on the key role of PI3K in other experimental models of immunological disease (reviewed in Refs. 38–41). PI3Kγ-dependent Regulation of Macrophage FcγRII and FcγRIII in Vitro and in Vivo—C5a is a major regulator of FcγR with induction of activating FcγRIII and suppression of inhibitory FcγRII (13), thereby tuning FcγR-mediated release of PMN recruitment factors in lung IC inflammation (42). Previous studies also suggested abrogation of C5aR signaling and impaired FcγRIII-mediated functions in mice lacking Go12, a selective upstream regulator of PI3Kγ in macrophages (14), implicating a specific involvement of PI3Kγ in FcγR regulation by these cells. We have used three distinct experimental strategies to determine the potential role of the class IB PI3Kγ isomorph in C5a- and IgG IC-induced FcγR regulation.

In the first approach, we performed in vitro reporter gene assays using the AM cell line MH-S and the recently characterized C5a-responsive FcγRII (729 to + 501) and FcγRIII (−808 to +18) gene promoters (16). Enhanced versus suppressed FcγRIII and FcγRI promoter activities in transfected MH-S cells were obtained by stimulating them with C5a for 4 h (Fig. 2A). The simultaneous positive and negative FcγRIII and FcγRII regulation was equally sensitive to inhibition with wortmannin or a PI3Kγ-selective inhibitor (5-quinoxalin-6-ylmethylenethiozolidine-2,4-dione) or an anti-C5aR mAb. As a negative control, the effect of PP2, an Src kinase inhibitor that does not interfere with C5aR signaling (16), was analyzed, and no inhibition was observed. In the second approach, we directly instilled 200 ng of C5a into the tracheas of WT B6 and PI3Kγ−/− mice and tested for in vivo changes of FcγR transcription in BAL-AM cells. As shown in Fig. 2B, both FcγRIIIα and FcγRIIIγ mRNA levels were up-regulated, whereas FcγRII γ transcription was suppressed within 2 h after application of C5a. This inverse regulation of FcγRII and FcγRIII was impaired in the absence of PI3Kγ (Fig. 2B). IC-induced FcγR mRNA (data not shown) and protein (Fig. 2C) regulation was also abrogated in PI3Kγ deficiency. In the third approach, AM cells were collected at 0 and 4 h after IgG IC challenge and analyzed by flow cytometry as described (13). Fluorescence-activated cell sorter analysis revealed IC-induced changes in increased staining of FcγRII (0-h versus 4-h groups, 297 ± 71 versus 912 ± 33 mean fluorescence intensity ± S.E.; p = 0.039) and reduced staining of FcγRI (0-h versus 4-h groups, 424 ± 37 versus 191 ± 23 mean fluorescence intensity ± S.E.; p = 0.049) in WT B6 mice, indicating that the observed changes in FcγR mRNA (13, 14) correlate with modulated FcγRI/III surface membrane expression. However, regulation of FcγR, which requires C5aR (13), was absent in PI3Kγ−/− mice (Fig. 2C).
Collectively, these data indicate that PI3Kγ regulates FcγR expression in response to C5aR activation in vitro and in vivo.

Impaired IC Inflammation but Normal C5a in PI3Kγ-decient Mice—Because PI3Kγ is an apparent critical mediator of both in vivo responses to C5a- and IC-induced FcγR modulation, we then asked whether PI3Kγ not only affects the expression level of FcγR but also regulates the subsequent FcγR-mediated production of factors that dictate PMN migration in lung disease. Whereas TNFα induces expression of adhesion molecules, thus promoting PMN migration more indirectly, MIP-2 and MIP-1α represent direct recruitment factors for PMN (42, 43). Both PMN influx and hemorrhage were blunted in PI3Kγ deficiency (Fig. 3, A and B), and IC-induced release of all mediators tested (MIP-2, MIP-1α, and TNFα) and KC (data not shown) was decreased to background levels (Fig. 3, D–F). Curiously, however, the chemotactic activity of BALF from IC-treated PI3Kγ−/− mice showed only partial reduction (Fig. 3C), indicating that most, but not all, acute changes associated with the lung Arthus reaction were dependent on PI3Kγ.

We have previously shown that IC activation of lung macrophages by FcγR triggers C5a production both in vitro and in vivo (14). To test the possibility that cell-derived C5a might be responsible for the remaining chemotactic activity seen in PI3Kγ−/− mice, BALF from both PI3Kγ−/− and wortmannin-treated mice was analyzed for C5a activity by Transwell chemotaxis assays using bone marrow PMN cells from C5aR−/− mice.

The difference in the percentage of C5aR−/− and C5aR+/+ PMN indicated the presence of bioactive C5a. In contrast to BALF from PI3Kγ-deficient animals (Fig. 4A), the C5a-depend-
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ent chemotactic activity detected in BALF of 4-h IC-treated WT mice was markedly suppressed upon PI3K inhibition (Fig. 4B), suggesting that generation of C5a critically involves a PI3K activity, which, however, is not related to PI3Kγ.

PI3Kδ-dependent C5/C5a in IC Inflammation—Because macrophages normally coexpress class IA PI3Kδ and class IB PI3Kγ (25) (data not shown), we analyzed PI3Kδ−/− mice to determine the possible contribution of PI3Kδ to the production of C5/C5a in IC inflammation. At 2 h after IgG IC challenge, BAL-AM cells displayed increased C5 mRNA synthesis in WT mice (p < 0.001 compared with Ab controls) (Fig. 5A). In contrast to both WT and PI3Kγ−/− mice (Fig. 5A), C5 mRNA levels remained largely unchanged in AM cells of IC-treated PI3Kδ−/− mice (Fig. 5B). To examine whether the changes in PI3Kδ-dependent C5 gene induction correlate with release of C5a in acute lung injury, BALF from WT and PI3Kδ−/− mice was assayed for the appearance of C5a. In contrast to BALF from WT littermates, C5a-dependent chemotactic activity detected in BALF from 2- and 4-h IC-treated PI3Kδ−/− mice was strongly decreased (Fig. 5C), indicating that PI3Kδ mediates generation of C5 and C5a by macrophages in lung IC inflammation.

Impaired IC Inflammation in PI3Kδ−/− Mice—In C5-defective Hc−/− mice, it has been shown that IC-mediated cytokine production depends on C5a (43), so we finally assessed the biological relevance of PI3Kδ-mediated C5a to the release of cytokines and chemokines as well as hemorrhage and neutrophil infiltration. The impaired production of C5a in PI3Kδ−/− mice (Fig. 5) seemed to correlate with reduced TNFα and MIP-2 mRNA levels in AM cells at 2 h of IC challenge compared with WT mice (data not shown), decreased appearance of these mediators in BALF (at 4 h) (Fig. 6, A and B), reduced synthesis of KC and MIP-1α (Fig. 6, C and D), and significantly lower levels of red blood cells (Fig. 6E) and PMN (Fig. 6F) in alveoli. Taken together, our results support a model of IC inflammation in which the cellular communication of FcγR and C5aR is coordinated by distinct class I PI3K members in the initiation of the inflammatory cascade (Fig. 7).

DISCUSSION

In this study, we have analyzed the role of class IA and IB PI3K signaling molecules in the Arthus reaction, the classical animal model of IC disease. Genetic deletion of PI3Kγ completely abolishes the inflammatory cascade with respect to C5aR-mediated FcγR regulation and subsequent cytokine/chemokine production, hemorrhage, and alveolar PMN transmigration, indicating that PI3Kγ is the pivotal downstream mediator of C5aR through which the C5aR-FcγR axis (13, 14) controls early neutrophil accumulation in the bronchoalveolar compartment. These data significantly extend previous findings that provided evidence for a role of PI3Kγ in PMN movement in chemokine-induced lung neutrophilia in vivo (44). Moreover, they support the concept that, in the complex situation of an inflammatory disease, a major function of the G-protein-regulated PI3Kγ is to transmit early C5aR-triggered signals in macrophages, thereby promoting a shift in the receptor balance between activating FcγRIII and inhibiting FcγRII that is essentially required for the recruitment of neutrophils via FcγR-mediated synthesis of various secondary mediators (including MIP-2, KC, MIP-1α, and TNFα). In addition, our analysis of general PI3K inhibition versus selective PI3Kγ deficiency also implicates the participation of an additional PI3K in the generation of a full chemotactic gradient that requires C5a.

PI3Kδ is the most prevalent class IA PI3K isofrom in leukocytes and controls a wide range of distinct immunological responses, such as antigen presentation, cytokine and chemokine secretion by natural killer/T cells, Th2-mediated inflammation, and many others (45–50). An important new mechanistic aspect of this study is the finding that PI3Kδ plays a non-redundant role in the initial phase of the lung Arthus reaction with a specific requirement of PI3Kδ for C5a production upstream of C5aR and PI3Kγ. As a consequence of impaired C5a, PI3Kδ−/− mice exhibit reduced cytokine production and PMN migration, suggesting that macrophage PI3Kδ is important for neutrophil recruitment in the initiation of the inflammatory response. A recent study in C5-defective Hc−/− mice has concluded that activation of C5 is central to the inflammatory reaction in IC-mediated disease (43). We found a similar protective phenotype in PI3Kδ deficiency, thus indicating that cellular activation of PI3Kδ is the relevant C5/C5a-generating mechanism in lung pathology. It is important to note, however, that it is at present not clear whether, in addition to macrophages, other cell types are involved in the PI3Kδ-mediated process of C5a production.

In summary, PI3Kγ−/− and PI3Kδ−/− mice allowed dissection of the signaling mechanisms of two major cooperating receptor systems, C5aR and FcγR, in immune inflammation. The results suggest that the different receptors have distinct PI3Kγ- and PI3Kδ-specific requirements. PI3Kγ is the central player in C5aR signaling that sets the threshold for FcγR activation. Conversely, both FcγR (8, 14) and PI3Kδ are critical for local C5 and C5a production. Our observations also indicate that the two class IA and IB PI3K molecules might be essential components of the immune response to pathogenic IC and, as recently discussed for rheumatoid arthritis (41, 51), may represent potential molecular targets for pharmacological intervention in inflammatory and rheumatic autoimmune diseases.

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