Class IB-Phosphatidylinositol 3-Kinase (PI3K) Deficiency Ameliorates IA-PI3K-Induced Systemic Lupus but Not T Cell Invasion

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Class I_B-Phosphatidylinositol 3-Kinase (PI3K) Deficiency Ameliorates I_A-PI3K-Induced Systemic Lupus but Not T Cell Invasion

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Class I PI3K catalyzes formation of 3-poly-phosphoinositides. The family is divided into I_A isoforms, activated by Tyr kinases and the I_B isoform (PI3Kγ), activated by G protein-coupled receptors. Mutations that affect PI3K are implicated in chronic inflammation, although the differential contribution of each isoform to pathology has not been elucidated. Enhanced activation of class I_A-PI3K in T cells extends CD4+ memory cell survival, triggering an invasive lymphoproliferative disorder and systemic lupus. As both I_A- and I_B-PI3K isoforms regulate T cell activation, and activated pathogenic CD4+ memory cells are involved in triggering systemic lupus, we examined whether deletion of I_B could reduce the pathological consequences of increased I_A-PI3K activity. I_B-PI3Kγ deficiency did not abolish invasion or lymphoproliferation, but reduced CD4+ memory cell survival, autoantibody production, glomerulonephritis, and systemic lupus. Deletion of the I_B-PI3Kγ isoform thus decreased survival of pathogenic CD4+ memory cells, selectively inhibiting systemic lupus development. These results validate the PI3Kγ isoform as a target for systemic lupus erythematosus treatment. The Journal of Immunology, 2006, 176: 589–593.

The PI3K are dual-specific lipid and protein kinases that participate in numerous cellular responses. The class I-PI3K are subdivided into class I_A and I_B. Class I_A-PI3K consists of three catalytic subunits, p110α, p110β, or p110δ, which form complexes with the p85 regulatory subunits, and are activated by tyrosine kinase receptor signaling. Class I_B is composed of the catalytic subunit p110γ, and is activated mainly by G protein-coupled receptors (GPCR)1 (1, 2). Mutations in the PI3K pathway are involved in tumor generation, as well as in chronic inflammatory lupus-like disease (3–5). Although PI3K is a promising therapeutic target, knowledge of isoform-specific functions remains limited.

Systemic lupus erythematosus (SLE) is a chronic inflammatory disease, characterized at early stages by an increase in autoreactive/memory CD4+ cells (6–12). Deregulated T cells help trigger polyclonal B cell activation, giving rise to generalized B cell expansion, hypergammaglobulinemia, and increased autoantibody production. Circulating anti-DNA Ab form complexes that are captured in kidney, activating the complement cascade. As disease progresses, T cells and macrophages infiltrate the kidney and amplify the local inflammatory response (8). At advanced stages, mesangial proliferation, vascular collapse, and immune complex accumulation in kidney result in glomerulonephritis (GN) and renal failure (6–9).

Deregulation of T cell homeostasis is a critical early event in SLE (10–12). Both I_A- and I_B-PI3K regulate T cell differentiation, activation, and survival. I_B-PI3Kγ-deficient mice show T cell differentiation defects and reduced mature T cell activation (13–15). Deletion of the I_B isoform PI3Kδ reduces T cell activation (16); the I_A isoforms PI3Kα and β may also affect T cell activation, although this remains untested because deficiency in these isoforms is lethal in embryos (17, 18). Enhanced activation of I_A isoforms by p65^pI3K transgene expression in T cells increases CD4+ cell differentiation (15). In mature T cells, p65^pI3K transgene expression enhances survival, triggering an invasive lymphoproliferative disease and systemic lupus (3). Deletion of the negative regulator of the class I PI3K pathway, the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10, also induces SLE-like disease, confirming the capacity of PI3K to trigger this pathology (5).

We previously showed that enhanced activation of I_A-PI3K compensates some T cell differentiation defects in PI3Kγ−/− mice (15). Using p65^pI3K transgenic (Tg) mice, we tested whether I_B-PI3Kγ deletion could reduce the invasion, lymphoproliferation, and systemic lupus development induced by increased I_A-PI3K activity in T cells. PI3Kγ deletion did not abolish lymphoproliferation or invasion, but diminished CD4+ memory cell survival, leading to amelioration of lupus and prolongation of mouse life span. As an increase in pathogenic CD4+ memory cells is a hallmark of multigenic murine and human lupus (6–12), selective inhibition of
memory cell survival by deletion of one PI3K isoform (Iγ-PI3Kγ) suggests a treatment for this disease. These observations contribute to understanding the specific functions of Iα- and Iγ-PI3K isoforms and their contribution to inflammation.

Materials and Methods

**Mice**

p65PI3Kγ-Tg (C57/BL6) and PI3Kγ-deficient (129sv) mice were described previously (3, 13). p65PI3Kγ mice were crossed with PI3Kγ-deficient mice. The F2 generation was produced by F1 × F1 mating; F2 progeny were used for experiments. Lupus-like disease in p65PI3Kγ Tg PI3Kγ+/− mice was indistinguishable from that of p65PI3Kγ-Tg mice (3). As controls, we used littermates that did not express the transgene and were PI3Kγ−/− or PI3Kγ+/+−, which presented no obvious differences. Offspring were analyzed by PCR. Mice were bred and maintained under specific pathogen-free conditions at the Centro Nacional de Biotecnología animal facility. The Consejo Superior de Investigaciones Científicas ethics committee approved the protocols used for experiments with mice.

**Flow cytometry and cell death analysis**

Spleen and lymph node cell suspensions were prepared; erythrocytes were lysed, and cells were counted. For surface staining, Abs were FITC, PE, or biotin conjugated, and cells were stained with saturating concentrations (4°C). Biotinylated Abs were developed with streptavidin-Spectral Red (Southern Biotechnology Associates). Abs used were CD3 (145-2C11), CD4 (L3T4, H129.19), CD8 (Ly-2, 53-6,7), CD44 (pgp1, IM7), and CD62L (all from BD Pharmingen). The Annexin V FITC kit was from Corixa. Cells were analyzed on an EPICS XL with System II software (Beckman Coulter). Statistics analyses were performed using the StatView 512+ program and the χ² test (www.physics.csbju.edu).

**Biochemical and serological analyses and histology**

Serum Ig and isotype-specific anti-dsDNA Ab were measured, as described (3). Urine protein levels were assessed with Medi-test Protein 2 Strips (Macherey-Nagel) every 15 days. Mice were examined daily; when severe SLE symptoms appeared, affected mice were killed and organs were collected for histology or flow cytometry analysis. Tissues were fixed in 4% Formalin in PBS until processing, as described (3).

**Results**

**Prolonged life span in p65PI3Kγ-Tg/PI3Kγ-deficient mice**

We previously showed that the increase in Iα-PI3K activity induced by p65PI3K transgene expression in T cells causes accumulation of CD4+ memory cells, an invasive lymphoproliferative disorder, and development of SLE-like disease; p65PI3Kγ mice die of renal failure (3). Iα- and Iγ-PI3K isoforms exhibit a partial functional compensation during thymic development (15). We thus examined whether Iγ-PI3Kγ deletion could reduce the disease generated by enhanced Iα-PI3K activity in mature T cells. p65PI3Kγ-Tg mice were crossed with PI3Kγ-deficient mice, and resulting progeny were examined. Mice were euthanized when they showed signs of disease (ascamation and proteinuria; data not shown). Histological examination showed that 80% of p65PI3Kγ-Tg/PI3Kγ+/− mice developed renal disease symptoms by 16 mo of age. In contrast, ~70% of p65PI3Kγ-Tg/PI3Kγ−/− mice showed no symptoms at this age and ~30% remained healthy at 20 mo, near the end of their natural life span (Fig. 1A; Table I). Compared with p65PI3Kγ-Tg/PI3Kγ−/− littermates, p65PI3Kγ-Tg/PI3Kγ+/− mice showed a significantly prolonged life span.

As p65PI3Kγ-Tg mice show lymphocyte accumulation with age, we examined spleen and lymph node cell suspensions. p65PI3Kγ-Tg/PI3Kγ−/− and p65PI3Kγ-Tg/PI3Kγ−/− littermates had significantly higher splenocyte numbers than control littermate mice, although numbers were slightly lower in p65PI3Kγ-Tg/PI3Kγ−/− mice (Fig. 1B). In flow cytometry, the cell composition of p65PI3Kγ-Tg/PI3Kγ−/− spleens was comparable to that of p65PI3Kγ-Tg mice (3), with a significantly larger CD4+ T cell population and a slightly larger CD11b+ population compared with controls (Fig. 1C).

Lymphoproliferation was also observed in lymph nodes (data not shown). T cells from p65PI3Kγ-Tg mice infiltrate many nonlymphoid tissues (3). Histological analysis of lung (Fig. 1D) and kidney (data not shown) indicated that PI3Kγ deletion does not reduce the magnitude of infiltrates in p65PI3Kγ-Tg mice. These infiltrates are enriched in T cells (3), and flow cytometry analysis of lung suspensions showed no significant differences between T cell infiltrates in p65PI3Kγ-Tg/PI3Kγ−/− and p65PI3Kγ-Tg/PI3Kγ−/− mice (Fig. 1E). Iγ-PI3Kγ deletion thus prolonged p65PI3Kγ-Tg mouse survival, but
did not abrogate T cell lymphoproliferation or invasion in these mice.

Reduced renal disease in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice

SLE-like disease development is accompanied by polyclonal hypergammaglobulinemia and proteinuria (3, 7). p65\textsuperscript{PI3K}\textsubscript{Tg} mice (3) developed proteinuria with age, which was low or absent in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} littermates (data not shown). Total anti-DNA Ab levels, as well as IgM, IgG1, IgG2a, and IgG2b levels, were reduced in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mouse serum compared with p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} littermates (Fig. 2A).

Histological examination of kidney sections showed renal lesions in most p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice, including frequent hyaline casts in tubules, increased mesangial cells, inflammatory infiltration, hypercellular glomeruli, thickening of capillary walls and vascular obliteration, at an intensity similar to that in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice (Fig. 2B). These signs indicate severe mesangio proliferative GN (Berden score grades 2–4) (19) (Fig. 2B). These features were reduced or absent in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice, whose GN scores ranged from 0 to 1 (Fig. 2B; Table I). Granular immune complexes were abundant in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice, and were minimal or absent in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice (Fig. 2C). Proteinuria, renal lesions, and immune complexes suggest renal failure as the cause of early death of p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mouse. We detected no gender differences. Renal disease was thus less severe in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice, despite the presence of invasive lymphoproliferation, indicating that PI3K\textsuperscript{−/−} deletion specifically hinders lupus development.

Reduction in CD4\textsuperscript{+} memory T cell numbers in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice

CD4\textsuperscript{+} memory T cell accumulation, a characteristic of systemic lupus, is found in p65\textsuperscript{PI3K}\textsubscript{Tg} mice (3). These cells are implicated in disease development (6, 10–12). As PI3K\textsuperscript{−/−} regulates T cell activation (14, 15), we examined whether PI3K\textsuperscript{−/−} deletion could reduce CD4\textsuperscript{+} memory cell numbers. Despite the fact that PI3K\textsuperscript{−/−} deletion did not significantly affect the memory T cell pool in non-Tg mice (Fig. 3A), both the proportion and absolute numbers of CD4\textsuperscript{+}CD44\textsuperscript{hi} and CD4\textsuperscript{+}CD62L\textsuperscript{low} memory cells were significantly reduced in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} compared with p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} (Fig. 3, A and B). As p65\textsuperscript{PI3K}-induced CD4\textsuperscript{+} memory T cell expansion involves reduction of cell death rates within this population (3), we examined whether PI3K\textsuperscript{−/−} deletion restores normal CD4\textsuperscript{+} memory T cell death rates in p65\textsuperscript{PI3K}\textsubscript{Tg} mice. CD4\textsuperscript{+}CD44\textsuperscript{hi} memory cell death in vivo was higher in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} compared with p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice (Fig. 3C). Accordingly, spontaneous death of cultured CD4\textsuperscript{+} T cells from p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice was also significantly higher than those of p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice (Fig. 3D). In addition, we observed reduced CD4\textsuperscript{+} T cell survival after PI3K\textsuperscript{−/−} deletion in non-Tg mice (Fig. 3D). Together, the findings show that amelioration of lupus disease in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice correlates with reduced CD4\textsuperscript{+} memory cell survival.

Table I. SLE-like disease in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} and p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Mice</th>
<th>p65\textsuperscript{PI3K} Tg/PI3K\textsuperscript{−/−} (n = 20)</th>
<th>p65\textsuperscript{PI3K} Tg/PI3K\textsuperscript{−/−} KO (n = 18)</th>
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<tr>
<td>Disease incidence at 16 mo (％)</td>
<td>80</td>
<td>33</td>
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<td>Healthy at 20 mo (％)</td>
<td>0</td>
<td>33</td>
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<td>Advanced GN (score 3–4) (％)</td>
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<td>16</td>
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<tr>
<td>Moderate GN (score 2–1) (％)</td>
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<td>33</td>
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</tr>
<tr>
<td>Normal kidney (％)</td>
<td>0</td>
<td>50</td>
<td></td>
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* Mice were analyzed at appearance of disease symptoms.
* Mice with no external signs of disease.

**FIGURE 2.** Reduced renal disease in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice. A, Anti-dsDNA total Ab, IgM, IgG1, IgG2a, and IgG2b in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} and p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mouse sera. Ab titers are represented as absorbance at OD492 nm at a 1/1600 serum dilution. Δ, Titer of a serum pool from littermate wild-type mice; ▲, mean Ab titer from groups of p65\textsuperscript{PI3K}\textsubscript{Tg} and MRL/lpr mice, which yielded similar results. Student’s t test p values are indicated. B, H&E-stained kidney sections from representative PI3K\textsuperscript{−/−} (normal) and p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice (GN score 3), and two p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice (GN score 1). C, FITC anti-IgG Ab stained IgG deposits in representative mice. IgG deposits are seen in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} mice and are notably reduced or absent in p65\textsuperscript{PI3K}\textsubscript{Tg}/PI3K\textsuperscript{−/−} kidney. Control non-Tg kidney is shown for comparison.
PI3Kγ DELETION AMELIORATES SYSTEMIC LUPUS

the pathological consequences of increased $I_A$-PI3K activity in mature T cells. $I_N$-PI3Kγ deletion did not abolish $I_A$-PI3K-induced T cell invasion or lymphoproliferation, but reduced CD4+ memory cell survival, as well as GN incidence and severity, prolonging p65$\text{PI3K}^\gamma$Tg mouse life span. Therefore, although $I_N$ is activated by Tyr kinases and $I_N$ by GPCR (1, 2), $I_N$-PI3Kγ deletion was capable of selectively ameliorating $I_N$-PI3K-induced lupus.

Normal quiescent T cells have low levels of PI3K lipid products, which increase only after cell activation (1, 2), $I_\gamma$-PI3Kγ-deficient mice show reduced T cell activation and impaired macrophage and neutrophil mobilization; however, they have a relatively normal hemopoietic cell composition in basal conditions (13, 14). Interference with PI3Kγ is thus predicted to induce minor side effects. Enhanced $I_N$-PI3K activation causes lupus (3–5). Moreover, endogenous PI3K activation was observed in a graft-vs-host-induced murine systemic lupus model (20) and in lupus-prone MRL/lpr mice (21). Our preliminary data suggest that PI3K activity is also increased in human SLE T cells (>75% of patients, $n = 17$, in progress). Increased basal PI3K activity may thus constitute a susceptibility factor for SLE, or be required for CD4+ memory cell maintenance. Validation of PI3Kγ as a target in systemic lupus, suggested by genetic PI3Kγ interference in this study, is further supported by pharmacological studies using PI3Kγ inhibitors in lupus-prone mice (21).

Chronic inflammatory autoimmune diseases are triggered by distinct factors, including defects in immune response down-regulation as well as central or peripheral tolerance defects, resulting in maintenance of autoreactive CD4+ memory cells (22). In the p65$\text{PI3K}^\gamma$Tg model, lupus is a consequence of excessive CD4+ T cell survival (3), resulting in CD4+ memory cell accumulation. Recovery from SLE-like disease following PI3Kγ deletion correlates with a reduction in CD4+ memory T cell numbers. As pathogenic CD4+ memory cells contribute to SLE development (10–12), the decrease in memory cells is probably a basic mechanism by which PI3Kγ inhibition ameliorates lupus. The decrease in CD4+ memory cells with helper activity is probably responsible for the diminished B cell activation, and may reduce macrophage and neutrophil activation. The studies presented nonetheless do not exclude a direct effect of PI3Kγ deletion on these populations.

No differences in disease development were observed between male and female p65$\text{PI3K}^\gamma$Tg mice (3). As higher female SLE incidence in other models correlates with a higher proportion of pathogenic CD4+ cells (10), the observation that the p65$\text{PI3K}^\gamma$ transgene enhances CD4+ cell survival similarly in males and females (3) explains the lack of gender-susceptibility differences. Similarly, PI3Kγ deletion reduced survival of CD4+ memory cells in a gender-independent fashion, explaining why both males and females recovered following PI3Kγ deletion.

Regarding specific and redundant $I_N$ and $I_B$ isoform functions, enhanced $I_N$-PI3K activity compensates the defective pre-TCR-triggered CD4+ CD8+ differentiation and TCR-induced CD4+ cell generation in $I_N$-PI3Kγ−/− mice. This suggests that the two isoforms cooperate to trigger T cell differentiation (15). Concurring with this, the PI3Kγ5−/− phenotype shows a striking T cell development blockade (23). In this study, we show that both $I_N$ and $I_B$ isoforms regulate CD4+ memory cell survival in mature T cells. Comparison of in vitro PI3Kγ−/− and PI3Kγ−/− T cell survival supports an independent role for PI3Kγ in CD4+ T cell survival. PI3K also regulates T cell activation in vitro and in vivo (14) (our data not shown).

Despite PI3Kγ involvement in T cell activation and survival, the proportion of memory T cells in non-Tg PI3Kγ−/− and PI3Kγ−/−...
mice was similar, suggesting that a homeostatic mechanism compensates the potentially reduced memory cell survival and generation in P13Kγ−/− mice. Similar homeostatic correction is found in the P13Kγ contribution to T cell differentiation. Although defective T cell differentiation is detected in newborn P13Kγ−/− mice, T cell populations are near normal in 15-day- to 1-mo-old animals, with only a modest reduction in peripheral CD4+ T cell numbers (15).

Our data show that P13Kγ deficiency reduces CD4+ memory T cell survival in p65ERK-Tg mice. Nonetheless, we cannot exclude that one effect of P13Kγ deletion in this model is a reduction in memory cell generation, as suggested by experiments in MRL/lpr mice (21). In these mice, P13Kγ inhibition also ameliorates lupus and reduces pathogenic CD4+ memory cell numbers (21). In MRL/lpr mice, however, the reduction in CD4+ memory T cells is not linked to variations in survival, because apoptosis is defective due to the lpr/Fas mutation (7). The reduction in CD4+ memory cells in P13Kγ inhibitor-treated MRL/lpr mice is thus probably the result from a reduction in memory cell generation.

Although the consequences of P13Kγ interference are similar regarding lupus amelioration and CD4+ memory cell reduction, inhibition of P13Kγ in MRL/lpr mice reduces hypercellularity more effectively, with a selectively greater effect on the CD4+ cell pool (21). Total CD4+ T cell numbers are also reduced in P13Kγ−/− mice (15). This effect is not seen in P13Kγ−/−/p65ERK-Tg mice, however, suggesting that enhanced IA activity has a unique function in inducing lymphoproliferation that is not counteracted by IA deficiency.

P13Kγ is involved in macrophage, neutrophil, and thymocyte migration (13–15). P13Kγ deletion, however, did not reduce T cell invasion induced by enhanced IA-PI3K activity. Accordingly, T cell homing to lymph nodes is only moderately affected by P13Kγ deletion (24). T cell invasion thus probably involves IA-PI3K activation through a Tyr kinase pathway, whereas thymus growth may require IA-PI3K activation through a GPCR. As for IA and IA isoform control of T cell activation and survival, either receptors that trigger these responses activate both isoforms, or distinct receptors that activate IA and II differ, cooperate in promoting CD4+ cell survival and activation.

The results show that whereas IA isoforms are dominant in the induction of lymphoproliferation and invasion, IA-PI3K deletion reduces CD4+ memory cell survival, even in the presence of active IA isoforms. As pathogenic CD4+ memory cell increase contributes to multiorgan murine and human lupus (6–12), the selective reduction of CD4+ memory cells and subsequent amelioration of systemic lupus upon IA-PI3Kγ deletion suggest that this isoform is a promising target for SLE treatment.

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Disclosures

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