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Enhanced Phosphoinositide 3-Kinase δ Activity Is a Frequent Event in Systemic Lupus Erythematosus That Confers Resistance to Activation-Induced T Cell Death

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Systemic lupus erythematosus (SLE) is a chronic inflammatory disease caused by the action of autoreactive T and B cells. Class I phosphoinositide-3-kinases (PI3K) are enzymes that trigger formation of 3-poly-phosphoinositides that induce cell survival. Enhanced PI3K activation is a frequent event in human cancer. Nonetheless, in a genetic model with enhanced activation of class Iγ PI3K in T cells, mice show a greater tumor index but die of a lupus-like disease. In this study, we studied the potential PI3K involvement in human SLE. The PI3K pathway was frequently activated in SLE patient PBMC and T cells (~70% of cases), more markedly in active disease phases. We examined the mechanism for PI3K pathway activation and found enhanced activation of PI3Kδ in SLE peripheral blood T cells. The magnitude of PI3K pathway activation in patients paralleled activated/memory T cell accumulation. We examined potential tolerance mechanisms affected by increased PI3K activity; SLE patients showed reduced activation-induced cell death of activated/memory T cells. Moreover, the defective activation-induced cell death in SLE T cells was corrected after reduction of PI3Kδ activity, suggesting that PI3Kδ contributes to induction of enhanced SLE memory T cell survival. These observations point to PI3Kδ as a target of clinical interest for SLE. The Journal of Immunology, 2011, 187: 000–000.

A common feature in murine and human SLE is the presence of increased numbers of autoreactive T and B cells and an accumulation of CD4+ memory cells (7, 8). Autoreactive T cells support B cell-mediated production of autoantibodies that form immune complexes; autoreactive SLE T and B lymphocytes affect a variety of organs, including skin, brain, and kidney (9). Circulating anti-DNA Abs form complexes that are captured in kidney, the most frequently affected organ, and activate the complement cascade. T cells and macrophages infiltrate affected organs and amplify a local inflammatory response (10). In kidney, at advanced disease phases, the mesangial proliferation, vascular collapse, and immune complex deposition result in renal failure (4, 11). De-regulation of T cell homeostasis is thus a critical event in SLE; distinct T cell defects have been reported in this disease, including altered TCR signaling, reduced IL-2 production, and COX-2 up-regulation (7, 12–16).

Class I phosphoinositide-3-kinases (PI3K) are heterodimeric enzymes comprised of a regulatory and a conserved p110 catalytic subunit that catalyzes formation of phosphoinositide-3,4-diphosphate and phosphoinositide-3,4,5-triphosphate (PIP3) (17). These lipids recruit proteins containing a pleckstrin homology domain (such as protein kinase B [PKB]) to the cell membrane, promoting cell survival (18). Generalized activation of PI3K in T cells triggers an accumulation of CD4+ memory cells and lupus-like disease in mouse, as shown by transgenic expression of an active allele of the p85 regulatory subunit (19), or by heterozygous loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which negatively controls PIP3 levels (20).

Class I PI3K are subdivided into p85/p10 heterodimers (p110α, p110β, or p110δ) that are mainly activated by tyrosine kinases (class Ia), and p87- or p101-p110γ heterodimers that are activated generally by G protein-coupled receptors (class Ib) (18). p110δ and p110γ are more abundant in hematopoietic cells, and their deletion affects the immune response (reviewed in Ref. 17). Deletion of p110δ reduces T cell activation and B cell differentiation.
whereas p110γ deletion impairs macrophage and neutrophil migration as well as T cell activation (21, 22). In the mouse, interference with p110γ ameliorates lupus-like disease by decreasing survival of CD4+ memory cells (23).

In this paper, we studied the contribution of PI3K to human SLE. We detected activation of the PI3K/PKB pathway, which correlated with an increase in the CD4+ memory T cell population in a significant proportion of SLE patients (70%); this phenotype was more pronounced in patients with active disease. SLE T cells presented an activation-induced cell death (AICD) apoptosis defect; reduction of p1106 activity corrected their AICD defect. Our results show that p1106 increased activity is a frequent event in inactive SLE and even more markedly in active SLE, pointing at inhibitors of this enzyme as a promising treatment for this disease.

Materials and Methods

Study subjects

Eighty-eight SLE patients, 20–58 y old and fulfilling at least 4 out of 11 American College of Rheumatology classification criteria for SLE were studied, as well as 45 approximate age- and sex-matched healthy donors (24–55 y old). SLE disease activity index (SLEDAI) scores from patients ranged from 0–44. Patients were classified according to their treatment as follows: untreated, treated with nonsteroid immunosuppressors alone (nonsteroid anti-inflammatory drugs [NSAID]; azathioprine or cyclophosphamide), treated with prednisone plus NSAID, or treated with prednisone plus NSAID and methotrexate. All patients were left untreated for a minimum of 24 h before blood extraction. We examined 12 rheumatoid arthritis patients. The La Paz Hospital Ethics Commission approved the study protocol and obtained informed consent.

Abs, cDNA, and cells

For Western blot (WB), we used rabbit polyclonal anti-phospho-Ser473–PKB unless indicated, Thr108–PKB, -p110α (C37FS8), -p110β (C33D4; Cell Signaling Technology), goat anti-PTEN, rabbit anti-p110γ (SC-7177; Santa Cruz Biotechnology), and rabbit anti-p110α (ab1678, Abcam) Abs. For immunopurification, we used the same Ab or anti-CD28 (Biosource International), -p110α (ab32401; Abcam), -FceRγ (Upstate), or -ZAP70 (Transduction Laboratories). Conjugated Abs to CD3, CD4, CD8, CD29, CD45RA, CD45RO, CD11b, CD64, CD66b, and CD19 were from BD Biosciences. T cells were activated with anti-CD3 (UCHT-1) and -CD28 (BD Pharmingen) mAb, anti-CD3 (OKT3; eBioscience) or phytohemagglutinin A (PHA; Invitrogen). PBMC were isolated and T cells purified as described (24). PMT2-KR-p110β and pcDNA3.1-KR-p110γ plasmids were donated by B. Vanhaesebroeck (Institute of Cancer, London, U.K.) and M. Wymann (University of Basel, Basel, Switzerland) and were subcloned in pEF-BOS XC. p110-cαx mutants were described (24).

Flow cytometry, WB, and PKB assays

To identify PBMC subpopulations, we studied samples from 55 SLE patients (21–58 y of age; SLEDAI 0–30) and 31 healthy donors (24–55 y old). Flow cytometry was performed using three-color immunofluorescence (IF) with a Coulter Epics XL-MCL flow cytometer (Coulter) (24). Extraction (in 1% Triton X-100 lysis buffer), WB, and PI3K assays were described (24). We used specific Abs (see above) to immunoprecipitate PI3K isomers from cell extracts (200 μg) prepared immediately after blood extraction. Immunopurified PI3K was resuspended in 45 μl 50 mM HEPES containing phosphoinositide-4,5-diphosphate (0.5 mg/ml; Sigma-Aldrich). The kinase reaction (final volume 50 μl) was initiated by adding 5 μl 10X kinase buffer (10 μCi [γ-32P]ATP, 100 mM MgCl2, and 200 μM cold ATP). Reactions were incubated (25 min, 37 °C) and terminated by addition of 1x RIPA (100 μl) and methanol/chloroform (1:1 v/v; 200 μl). Phospholipids were resolved in silica gel 60 plates (Merck) (pretreated with 1% potassium oxide) with glacial acetic acid/H2O/propyl alcohol (4:3:1 v/v/v). Radioactive products were visualized by autoradiography and quantified with Imagel (National Institutes of Health); specific PI3K activity was calculated by dividing PIP, signal intensity by enzyme levels determined in total cell extracts (50 μg) by WB and quantitated with Imagel (National Institutes of Health).

DNA synthesis, anergy, apoptosis assays, and transcription

Anergy was induced as reported (25). Plates (24-well; Falcon/BD Biosciences) were coated with 1 μg/ml (0.3 μl/well) goat anti-mouse IgG (Sigma-Aldrich) in 150 mM Tris-HCl (pH 8.8) (overnight). 4°C. Plates were washed and coated with anti-CD3 (OKT3) as above. Cells (6 × 105/well) were added in RPMI 1640/10% FCS in triplicate. After 7 d, cells were transferred to uncoated 24-well plates and incubated 24 h, then replated (1 × 106/well) in 96-well plates precoated with anti-CD3 (UCHT1; 1 μg/ml) as above. DNA synthesis was examined after 72 h by adding [3H]thymidine (25 μCi/ml; Amersham Biosciences) for the last 12 h; for Fig. 8, 96-well plates were coated with anti-CD3 and -CD28 (0.15 μg/ml each) (24). For spontaneous and AICD, a set of SLE patients (SLEDAI 0–12; 20–45 y old) and healthy donors (25–55 y old) were analyzed in parallel. T cell lines were established from PBMC by activation with 1% PHA in RPMI 1640/10% FCS (72 h) and subsequent expansion in the presence of IL-2 (30 U/ml). After 13 d, 1.5 × 105 cells were transferred to untreated 96-well culture plates (for spontaneous cell death) or plates precoated with 0.3 μg/ml anti-CD3 mAb (OKT3) in 0.1 ml/well (for AICD). After 72 h, cells were stained with Annexin/propidium iodide (23). Jurkat cells were electroporated and examined with cDNA at 290 V and 975 μF, as reported (26).

Indirect IF

PBMC were washed twice with incomplete RPMI and resuspended in 1 ml PBS-EDTA. For IF, we purified CD4+ cells by negative selection; PBMC were stained with PE-conjugated anti-CD8 plus FITC–anti-CD5RO or, alternatively, with PE–anti-CD8 plus FITC–anti-CD45RO. Unlabeled Abs were sorted on an Epics Altra (Beckman Coulter), then stained to confirm membrane or naive CD4+ T cell purification; the proportion of CD8+ cells was always <1%. Purified cells (5 × 105) were resuspended in RPMI with 10% individual donor serum (10 min) and then plated on poly-l-lysine-coated glass slides using a cytospin. Cells were fixed in 4% paraformaldehyde/PBS (5 min), washed twice in PBS, permeabilized by incubation with 0.3% Triton X-100 in PBS (5 min), and blocked in 0.01% Triton X-100 in PBS containing 1% BSA/10% goat serum (1 h, room temperature). Cells were incubated (overnight, 4°C) with anti-Ser473–p-PKB and -CD3 (UCHT-1) or -CD19 (Santa Cruz Biotechnology), labeled with Alexa Fluor 488-goat anti-rabbit IgG and Cy3-goat anti-mouse IgG Ab, and stained/mounted with Hoechst 33258 (10 μg/ml) in 33% glycerol/PBS. Confocal microscopy and double-fluorescence analysis were performed with a Fluoview1000 (Olympus) or Zeiss Axiosvert LSM 510 (Zeiss) confocal microscope.

Statistics and quantitation

Data are presented as mean ± SEM. Where mentioned, unpaired two-tailed Student t test was included with Welch’s correction; the χ2 test and ANOVA were also used (indicated). Correlation significance was established with Pearson’s correlation coefficient. Statistics were performed using GraphPad Prism 5.0 software (GraphPad). Significance was defined as p < 0.05. Gel bands and fluorescence intensity were quantitated with ImageJ software (National Institutes of Health). All quantitation was performed using low-exposure film (in the linear range); for quantitation of IF images, all images were acquired in the same conditions.

Results

The PI3K/PKB pathway is frequently activated in PBMC from SLE patients

We compared the activation status of the PI3K pathway in approximate age- and sex-matched healthy donors and SLE patients (untreated for at least 24 h prior to blood extraction). We prepared PBMC extracts immediately after purification and determined activation of the PI3K effecter PKB by evaluating p-PKB levels in WB (23). Most control individuals had low p-PKB levels; in contrast, ∼70% of the SLE patients showed high p-PKB levels (Fig. 1A), within the range of p-PKB levels observed after T cell activation (Supplemental Fig. 1A). We quantitated p-PKB levels and normalized them to PKB. The p-PKB/PKB ratio in SLE patients (n = 48) and controls (n = 30) indicated that PBMC from SLE patients had higher p-PKB levels than healthy donors (3.7 ± 0.6 versus 1.0 ± 0.1, respectively; Fig. 1B). High p-PKB/PKB levels were found in untreated patients as well as in those treated with nonsteroid or steroid immunosuppressors or combinations; PI3K pathway activation is thus intrinsic to disease.

SLE is a chronic disease that remains inactive for long periods, separated by phases of high activity; these can be assessed using
SLEDAI (27, 28). To determine whether there is a relationship between PI3K pathway activation and disease activity, we studied the correlation between increased p-PKB levels and high SLEDAI scores using a cutoff value of 4 for active and inactive cases (29). We analyzed p-PKB/PIKB ratios in patients with active (SLEDAI ≥4; n = 27) and inactive SLE (n = 21) (Supplemental Table I). Although p-PKB values were higher in SLE patients than controls and higher in active than inactive SLE (3.9 ± 0.6 versus 2.1 ± 0.5, respectively; p = 0.03; Fig. 1B), the SLEDAI did not correlate well with the p-PKB/PIKB ratio (not shown). This imperfect correlation might be due to the fact that the SLEDAI score is based on symptoms rather than on molecular defects. Rheumatoid arthritis patients did not show increased p-PKB/PIKB ratios (Supplemental Fig. 1C). PI3K/PIKB pathway activity is thus elevated in PBMC from SLE but not from rheumatoid arthritis patients.

The PI3K/PIKB pathway is activated in SLE naive and memory T cells

T lymphocytes are the most abundant cell subpopulation in PBMC (70%); the increase in PKB activity might reflect PI3K pathway activation in T cells. To examine this, we purified T cells from a larger blood volume in a group of patients. Patients showed parallel results in T lymphocytes and PBMC (Fig. 1C), indicating that the enhanced PI3K/PIKB pathway activation in PBMC is due at least in part to pathway activation in T cells.

According to their previous Ag exposure, peripheral T cells are divided into naïve T cells, which have not previously encountered Ag, and memory cells, which have been activated and differentiate into long-lasting, more efficient Ag-responsive cells (7, 15). SLE disease is maintained throughout life due to the presence of autoreactive/memory T cells (3, 12, 13). To determine whether the increase in PI3K activity is a consequence of disease (present only in activated/memory cells) or an intrinsic defect in naïve T cells prior to autoantigen stimulation, we examined pathway activation in both cell subsets.

We compared p-PKB IF staining in CD4+CD45RA+ or CD45RO+ cells, the former (CD45RA+) expressed in naïve T cells and the latter in memory T cells (30). p-PKB levels were increased both in naïve and memory T cells from SLE patients (Fig. 2). Whereas a certain level of p-PKB was found in the nucleus in control and SLE T cells, SLE naïve T cells, but not control naïve T cells, showed a positive p-PKB signal at the cell membrane (Fig. 2). In addition, memory cells had higher p-PKB levels than naïve cells in both healthy individuals and SLE patients, but SLE memory cells presented significantly higher p-PKB levels than memory cells from controls (Fig. 2). Activation of PI3K/PIKB reduces the T cell activation threshold (19); the presence of active-PKB in naïve T cells from SLE patients might contribute to facilitate activation by autoantigens and disease development.

The low proportion of B cells (7–10%) in the small blood sample available from patients did not permit WB analysis; we thus examined PI3K/PIKB pathway activation by IF. B cells were simultaneously stained with an anti-CD19 mAb and an anti-p-PKB Ab. SLE B cells had higher p-PKB levels at the cell membrane than control B cells (Supplemental Fig. 1C). Quantitation of p-PKB signal intensity (in the same image collection conditions) nonetheless showed that naïve and memory T cells have a higher p-PKB signal intensity than B cells (Fig. 2, Supplemental Fig. 1C).

The memory T cell population is increased in SLE

Expression of an activated PI3K allele as a transgene in murine T cells increases the proportion of CD4+ memory cells and induces development of a lupus-like disease (19). To test for a potential relationship between PI3K and memory cell expansion in the human disease, we compared PBMC populations in healthy donors and SLE patients

SLE patients showed a moderately increased proportion of B cells (~3%) and a modest decrease in T cells (Supplemental Fig. 2A). Within T cells, SLE samples showed an increase in the proportion of CD8+ and a decrease in the proportion of CD4+ T cells compared with controls (Supplemental Fig. 2A). The reduction in the proportion of CD4+ T cells is probably due to the presence of anti-CD4 lymphocyte Abs in human SLE patients (31); neutrophils and macrophages were increased in ~20% of the patients (10).

To evaluate memory cell populations, we examined CD45RA+ and CD45RO+ cells (30). Both CD4+ and CD8+ T cell subsets had...
Altered CD4+ memory/naive ratio correlates with high p-PKB which reduces CD4+ cell numbers in SLE patients (31). We used lymphocyte autoantibody, particularly in active disease phases, to increase in parallel with p-PKB levels in active SLE (Supplemental Fig. 2B). p-PKB signal intensity was quantitated in membrane, cytosol, or nucleus (mean ± SEM; n = 25 cells/patient). Scale bars, 5 μM. χ² test, *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 2.** p-PKB levels are increased in SLE naive and memory T cells. Sorted CD4+CD45RO+ or CD4+CD45RA+ T cells from eight active SLE patients or healthy controls were stained with Hoechst 33258, anti-CD3, and –p-PKB Ab (representative images). p-PKB signal intensity was quantitated to increase in parallel with p-PKB levels in active SLE (Supplemental Fig. 2B). This is probably caused by the presence of anti-CD4–lymphocyte autoantibody, particularly in active disease phases, which reduces CD4+ cell numbers in SLE patients (31). We used memory/naive cell proportions to compare all patients, which showed enhanced naive to memory cell differentiation in SLE, independently of the presence of anti-CD4–lymphocyte Ab.

The memory/naive cell ratio must be maintained for T cell homeostasis; an increase in memory/naive ratio is an indication of disease onset (19, 33). The memory/naive cell ratio in controls was ∼1 for CD4+ and ∼0.3 for CD8+ cells (33). In agreement with the high proportion of memory cells found in SLE, memory/naive ratios were higher in SLE CD8+ and CD4+ cells and markedly higher in active SLE CD4+ cells (Fig. 4A).

**Altered CD4+ memory/naive ratio correlates with high p-PKB levels in active SLE.**

We examined whether PI3K/PKB pathway activation in SLE correlated with an accumulation of memory cells. No correlation was found between p-PKB levels and CD8+ memory cells (20–40% of total memory cells). Nonetheless, although absolute CD4+ memory cell numbers vary among patients due the presence of anti-CD4 autoantibody (31), CD4+ memory cell numbers tended to increase in parallel with p-PKB levels in active SLE (Supplemental Fig. 2C). Moreover, p-PKB levels correlated well with the CD4+ memory/naive ratio in active SLE (Fig. 4B). p-PKB levels thus increased in parallel with the proportion of memory cells as SLE progressed; in active SLE, high p-PKB levels correlated with high CD4+ memory/naive ratios.

**p110δ is frequently activated in SLE.**

PI3K/PKB pathway induction can be achieved either by enhanced activation of a PI3K isoform or by deletion of the PIP3-phosphatase PTEN. We analyzed whether enhanced PI3K/PKB pathway activation in SLE patients correlated with reduced PTEN levels, but found no differences in PTEN levels compared with controls (Supplemental Fig. 3A). To establish whether activation of a p110 isoform might be responsible for p-PKB enhancement in human SLE, we compared the activity of the two most abundant hematopoietic PI3K isoforms, p110γ and p110δ, in healthy controls and SLE patients.

PI3K isoforms were immunopurified from cell extracts prepared immediately after blood extraction. We determined enzyme expression levels in total extracts by WB and measured PIP3 production using in vitro kinase assays; specific PI3K activities were calculated considering PIP3 production and enzyme levels. Each assay was controlled by parallel examination of p110δ activity was similar in controls and patients; in contrast, specific p110γ activity was increased in SLE and more markedly in active SLE (Fig. 5). Quantitation of p110γ and p110δ levels and activity in a larger patient cohort showed that differences in p110γ activity were not significant, whereas more than half of the SLE patients showed increased p110δ activity, which was more marked in active SLE cases (Fig. 6A). We found that p110δ activation was independent of patient treatment (not shown).

PI3K associates to transmembrane receptors following stimulation (17). To confirm that p110δ activity was increased in active SLE T lymphocytes, we sought a T lymphocyte receptor that preferentially bound p110δ. Control cells were activated with the pan-T cell mitogen PHA, which binds to all glycosylated receptors, and
tested p110 association with receptors such as FcεRIγ-chain, CD3, and CD28 or to the T cell intracellular kinase ZAP70 (16, 34).

FcεRIγ-chain associated the greatest amount of p110 (Fig. 6B). FcεRIγ-chain expression is increased in SLE (35) and could contribute to p110 activation in patients. Because control T cells had very low FcεRIγ-chain levels, however, we could not use this receptor to compare p110 activation in SLE patients and control individuals. ZAP70 also associated to p110 even before cell stimulation and to a greater extent than p110ɣ (Fig. 6B). ZAP70-associated PI3K activity was also increased in approximately two thirds of SLE samples and was more marked in active SLE (Fig. 6C), confirming that p110 activation contributes to PI3K/PKB activation in SLE (Fig. 6C).

Anergy is similar in control and in SLE T cells

Several mechanisms safeguard mammals from T cell-mediated autoimmunity. Thymic negative selection induces deletion of developing autoreactive T cells (central tolerance). This process is complemented by peripheral tolerance mechanisms that include the action of regulatory T cells (Treg), induction of anergy (when antigenic stimulation is incomplete), and induction of apoptosis (25, 36–38). In the case of apoptosis, two processes contribute to downregulation of the immune response: spontaneous apoptosis, which occurs when cytokine levels decrease at the end of an immune response (26), and AICD, which occurs when already activated T cells are restimulated with Ag (such as a self-antigen) (38).

Central tolerance shows no defects in murine lupus models (39); the contribution of Treg in SLE is debated (3, 37). We examined anergy by stimulating control and SLE-derived T cells with anti-CD3 (without anti-CD28 Ab) (34). In these conditions, secondary stimulation results in defective proliferation of T cells, which is corrected by IL-2 addition (25); we found no differences in anergy induction between control and SLE patient T cells (Fig. 7A).
AICD defect in SLE

Considering that PI3K activation is a major pathway in induction of cell survival (17), we next examined apoptosis. We analyzed spontaneous death and AICD following previously reported protocols (38, 40). We stimulated purified lymphocytes in a primary immune response and cultured them with IL-2 for 13 d. Cells were then deprived of IL-2 (spontaneous death) or restimulated with anti-CD3 (AICD). We detected no significant difference in the proportion of spontaneous apoptosis in T cells from SLE patients and controls (Fig. 7B). To evaluate apoptosis of activated cells, we calculated the ratio between the proportion of activated cells that underwent apoptosis after subsequent activation (AICD) and after growth factor deprivation (spontaneous apoptosis); a value of 1 indicates that no notable AICD is induced. SLE patients showed a pronounced AICD defect, more marked in active SLE (Fig. 7C). Defects in AICD were found in treated and untreated patients (not shown). Because AICD takes place in previously activated cells, defective AICD is a mechanism for memory cell accumulation in SLE.

AICD defect is induced by increased PI3K pathway activation

Activation of PI3K is sufficient to increase cell survival (19); defective apoptosis in SLE T cells could thus be caused by enhanced p110δ activity. To test whether alterations in PI3K activation lead to AICD resistance, we tested the consequences on this process of expressing inactive p110δ or p110γ mutants (KR-p110δ, KR-p110γ) (21, 24). We examined AICD in a human T cell leukemia cell line (Jurkat) that lacks PTEN expression and shows generalized activation of the PI3K pathway (41). Jurkat cells were electroporated with cDNA encoding control vector, KR-p110δ or KR-p110γ (24 or 48 h), or with a constitutive active p110δ-caax mutant (Fig. 7D, 7E). Active p110δ expression moderately increased p-PKB and reduced AICD, whereas inactive p110δ or p110γ reduced p-PKB levels and increased AICD in Jurkat cells (Fig. 7D, 7E). We confirmed these results using selective PI3K inhibitors: AS252424 for p110γ, IC87144 for p110δ, and Ly294002 as a pan-p110 inhibitor (42, 43). AS252424 and IC87144 selectively inhibited...
Inhibition of p110γ or p110δ reduced p-PKB levels and increased AICD in Jurkat cells (Fig. 7E), confirming that PI3K pathway activation modulates AICD. The recovery of AICD by both p110δ and p110γ inhibitors (or KR-mutants) is explained by the lack of PTEN in Jurkat cells that enhances the action of all PI3K isoforms.

Inhibition of p110δ rescues the AICD defect in SLE

Based on these observations, we hypothesized that defective apoptosis in SLE T cells might be caused by enhanced p110δ activity. To test this possibility, we selectively inhibited p110δ or p110γ prior to AICD induction in activated T cells from controls and SLE patients. p110δ and p110γ inhibitors have an effect similar to that of gene mutation in the mouse (44–46), but permit selective inhibition of each isoform at the time of AICD induction. General PI3K inhibition using Ly294002 increased AICD in controls and SLE (Fig. 8A, gray circles); inhibition of a single isoform with selective inhibitors did not significantly affect AICD in controls (Fig. 8A). In contrast, in SLE T cells, inhibition of p110δ but not of p110γ restored AICD (Fig. 8A). Selective inhibition of p110δ therefore corrected the AICD defect of active and inactive SLE patients. Involvement of p110δ activity in AICD regulation in SLE patients does not exclude the implication of additional PI3K isoforms in AICD control in human T cells, as generalized PI3K inhibition (with Ly294002) increased AICD in controls and patients. The selective action of p110δ inhibitors in AICD in SLE T cells is in agreement with the selective enhanced activation of this isoform in SLE.

A potential undesired effect of blocking PI3K isoforms is a reduction in primary T cell activation. We measured anti-CD3 plus anti-CD28–induced T cell activation in normal control cells in the presence of p110 inhibitors. At the highest doses (5–

FIGURE 6. Increased PI3Kδ activity is observed in active SLE. A. Cumulative data for quantitated p110γ or p110δ levels and sp. act. in PBMC from controls and patients with inactive and active SLE, as in Fig. 5. Graphs (right panels) show the mean of activity curves from stimulated controls as in Fig. 5 (n = 6). B. Representative WB of p110γ and p110δ coimmunoprecipitation with various anti-receptor or -ZAP70 Abs from extracts of PHA-activated PBMC (5 min). Right panel shows ZAP70 immunoprecipitation and WB for PI3K isoforms. C, PI3K assays in immunoprecipitates of ZAP70 and p110δ. WB shows protein levels in cell extracts (bottom panel). The p values were calculated as in Fig. 1.
10 μM), both AS252424 and IC87144 reduced T cell activation (Fig. 8B), as reported (21, 45); nonetheless, in the 0.5 μM range, the p110δ-specific inhibitor restored AICD in SLE T cells (Fig. 8A), but did not greatly affect normal T cell division (Fig. 8B). Partial p110δ inhibition thus restored AICD in SLE without markedly disrupting Ag-induced primary activation of normal T cells.

**Discussion**

PI3K is a major participant in the induction of cell survival; mutations in this pathway are frequent in human cancer. In the mouse, however, PI3K activation in vivo not only increases cancer susceptibility, but also reduces lifespan by triggering a lupus-like disease (19, 20). We examined the potential involvement of the PI3K pathway activation in human SLE and show that ~70% of SLE patients exhibit enhanced PI3K/PKB pathway activation in PBMC and T cells. This increased PI3K pathway activation in SLE appears to be caused by enhanced activity of the hematopoietic p110δ isoform. SLE patients also showed a frequent defect in AICD that was corrected by inhibition of p110δ. Activation of the PI3K/PKB pathway correlated with an increase in the proportion of CD4+ memory cells, a hallmark of murine and human SLE (7, 8). These results point at enhanced p110δ activity as a cause for the accumulation of long-lasting memory cells in SLE patients, because p110δ inhibition corrected the apoptosis defects of activated/memory SLE T cells.
We examined the mechanism of PI3K/PKB pathway activation by analyzing the kinase activity of the hematopoietic PI3K isoforms (p110α and p110γ) and the expression levels of the negative pathway regulator PTEN phosphatase. PTEN levels were unaltered (0 out of 13 SLE cases examined), but p110α kinase activity was frequently enhanced in SLE, suggesting that enhanced p110α activity is a mechanism underlying the increased PI3K/PKB activity in this disease. Activation of p110α, AICD defects, and CD4+ memory cell accumulation were found in inactive and active SLE patients, although all these phenotypes and the memory/naïve ratio increase were significantly higher in active SLE.

To determine whether PI3K/PKB pathway activation was found selectively in the cell population responsible for disease maintenance (long-lasting autoreactive/memory cells) or was also found in virgin T cells, we examined activation of this pathway in both populations by IF of the PI3K effecter PKB. The presence of activated PKB in naïve cells suggests that although p-PKB levels are higher in active phases (when autoreactive cells are reactivated), pathway activation is intrinsic to disease susceptibility, as it is found in cells that have not encountered Ag. Because PI3K activation enhances survival and reduces the activation threshold for T cells (18–20), PI3K/PKB activation in naïve cells might facilitate their activation by autoantigens and, in turn, disease generation. As mentioned above, p110α activity levels were even greater in patients with active SLE. One of the defects defined in human SLE T cells is the frequent replacement of the CD3 receptor ε-chain with the FceRy-chain (35), which associates p110ε (Fig. 6). In active SLE phases, PI3K activation might be increased due to reactivation of the TCR and associated FceRy in autoreactive cells.

We found no direct correlation of SLEDAI and PI3K/PKB activation. This lack of correlation could be due to the fact that the SLEDAI score is symptom-based rather than on molecular defects. SLE patients, particularly those with active disease, nonetheless had higher p-PKB values than controls. Comparison of p-PKB levels with memory cell accumulation showed that CD4+ memory cell number increased in parallel with p-PKB levels in SLE. Correlation was better when compared p-PKB levels and CD4+ memory/naïve T cell ratio in active SLE, as the memory cell proportion was a better indicator of the actual naïve to memory cell differentiation in SLE, independent of the potential presence of anti-CD4 autointobody (31). p-PKB levels thus increased in parallel to the proportion of CD4+ memory cells in active SLE. Several T cell tolerance defects have been described in SLE. We concentrated on apoptosis, as a large percentage of patients showed reduced apoptosis (AICD), and PI3K activation triggers cell survival (18). Other defects have been reported: Treg involvement in SLE (3, 37), anergy defects (47, 48), and the contribution of Th-17 cells (49). Despite potential involvement of additional tolerance mechanisms in SLE, our findings show that AICD is defective in this disease and corrected by p110α inhibition.

This study indicates the need to examine human samples in addition to mouse models. Although PI3K activation correlates with increased CD4+ memory cell population in both species, murine lupus development depends on p110γ activity (19, 23), whereas in man, PI3K/PKB pathway activation correlated with increased p110α activation. Our findings suggest the potential clinical use of p110α inhibitory compounds in SLE. The correction of AICD in SLE T cells by p110α inhibition indicates that this treatment might reduce autoreactive/memory cells in these patients. Clinical application is facilitated by the availability of p110α inhibitors, currently in clinical trials for chronic myeloid leukemia (44). Soond et al. (45) recently showed that p110α inhibition reduces naïve and memory T cell activation at high doses; however, we show that partial p110α inhibition normalized AICD in SLE T cells, whereas it only moderately reduced primary activation of healthy donor T cells (Fig. 8). p110α inhibition might also be beneficial in mitigating SLE symptoms by reducing B cell survival (44), and it reduces IgE-to-IgG class switch (46). PI3K activation regulates COX-2 levels (47), which are frequently increased in SLE (48); P3K inhibition could also ameliorate SLE by reducing COX-2 levels.

A large percentage of SLE patients (~70%) showed enhanced PI3K/PKB activation; activity was higher in active phases and correlated with an increased proportion of CD4+ memory T cells. The mechanism for the enhanced PI3K/PKB pathway activation in SLE was an increase in p110α activity, which was found in patients with inactive and active disease. We propose that increased p110α activity promotes enhanced survival of memory cells, contributing to their long-term survival and accumulation in SLE. AICD, which affects only cells previously exposed to Ag, was defective in SLE T cells and was corrected by p110α inhibition; this treatment might thus reduce long-term survival of the pathogenic/memory cell population in all patients. In addition, as disease reactivation produces higher p110α activity peaks, p110α inhibition might also be a treatment for active phases.

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