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CUTTING EDGE

Cutting Edge: Lentiviral Short Hairpin RNA Silencing of PTEN in Human Mast Cells Reveals Constitutive Signals That Promote Cytokine Secretion and Cell Survival[1]

Yasuko Furumoto, Steve Brooks,* Ana Olivera,* Yasuomi Takagi,‖ Makoto Miyagishi,¶ Kazunari Taira,¶ Rafael Casellas,† Michael A. Beaven,§ Alasdair M. Gilfillan,‡ and Juan Rivera[2]**

***Engagement of the FcεRI expressed on mast cells induces the production of phosphatidylinositol 3, 4, 5-trisphosphate by PI3K, which is essential for the functions of the cells. PTEN (phosphatase and tensin homologue deleted on chromosome ten) directly opposes PI3K by dephosphorylating phosphatidylinositol 3, 4, 5-trisphosphate at the 3′ position. In this work we used a lentivirus-mediated short hairpin RNA gene knockdown method to study the role of PTEN in CD34$^+$ peripheral blood-derived human mast cells. Loss of PTEN caused constitutive phosphorylation of Akt, p38 MAPK, and JNK, as well as cytokine production and enhancement in cell survival, but not degranulation. FcεRI engagement of PTEN-deficient cells augmented signaling downstream of Src kinases and increased calcium flux, degranulation, and further enhanced cytokine production. PTEN-deficient cells, but not control cells, were resistant to inhibition of cytokine production by wortmannin, a PI3K inhibitor. The findings demonstrate that PTEN functions as a key regulator of mast cell homeostasis and FcεRI- responsiveness. The Journal of Immunology,* 2006, 176: 5167–5171.**

There is increasing evidence that key steps in mast cell activation are mediated through the enzymatic activity of PI3K (1–4), whose product, phosphatidylinositol 3,4,5-trisphosphate (PIP_3),[3] binds to the pleckstrin homology domain of various signaling proteins, allowing their activation and targeting to the membrane and cytoskeleton. Because of the diversity of signaling proteins regulated by PIP_3, this lipid has broad effects on cell signaling and function. For example, mutation of the p110δ catalytic subunit of PI3K (4) and the genetic deletion of the Fyn kinase or of the adapter Gab2 (both of which regulate PI3K activity) (2, 3) significantly impaired FcεRI-mediated mast cell responses. In contrast, genetic loss of Lyn kinase or SHIP-1 (a 5′-phosphatase of PIP_3) increased intracellular PIP_3 levels (3, 5) and augmented FcεRI-mediated mast cell responses (5, 6). Thus, understanding how PIP_3 levels are regulated and what responses might be most sensitive to this lipid should provide new insights on its importance in cellular function.

PTEN (phosphatase and tensin homologue deleted on chromosome ten) opposes PI3K function by dephosphorylating the 3′ position of PIP_3. PTEN is a known tumor suppressor and a key regulator of cell growth and apoptosis (7). Although PTEN knockout mice are embryonic lethal, *PTEN*$^{+/-}$ mice develop an autoimmune disorder characterized by increased numbers of activated T cells and polyclonal lymphoid hyperplasia (8), demonstrating its importance in immune cell regulation.

To address the question of whether the aforementioned increase in PIP_3 was key in the hyperresponsiveness phenotype of Lyn- and SHIP-null mast cells (3, 5), we down-regulated PTEN expression in mast cells. Human mast cells provided the most suitable model for our studies due to their slow proliferation and nondetectable levels of PI3K activity in resting conditions (9). However, the genetic manipulation of these cells has been difficult to achieve. We speculated that the HIV-related lentivirus might prove useful in gene manipulation of these cells. We coupled this vector technology with the introduction of short hairpin RNA (shRNA) for sequence-specific posttranscriptional gene silencing. Using this approach, we now find that PTEN is a key regulator of mast cell homeostasis and function.

Materials and Methods

Antibodies and reagents

All Abs and reagents used in this study have been described elsewhere (3, 6, 9, 10). Biotinylated human IgE was obtained as described (9). Streptavidin (SA) was from Sigma-Aldrich. Secondary Abs were previously described (10).

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[3] Abbreviations used in this paper: PIP_3, phosphatidylinositol 3,4,5-trispohosphate; ATF2, activating transcription factor 2; HMC-1, human mast cell line 1; HuMC, human mast cell; IKK, IκB kinase; LAT, linker for activation of T cells; PLCγ, phospholipase Cγ; PTEN, phosphatase and tensin homologue deleted on chromosome ten; SA, streptavidin; shRNA, short hairpin RNA.

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Lentivirus shRNA vector construction and gene transduction

The entry vector (pEnter/U6) containing a U6 promoter, a double strand oligonucleotide, and a polymerase III terminator was used to transfer the U6 RNAi cassette into the lentiviral expression plasmid (pLenti6/BLOCK-iT-DEST) using Gateway Technology (Invitrogen Life Technologies). Recombination was performed with the pENTR/U6 entry construct and pLenti6/BLOCK-iT-DEST to generate the pLenti6/BLOCK-iT. The sense and antisense oligonucleotide sequence for construction of four PTEN (GenBank accession number NM_000314) shRNAs were as follows: PTEN no. 1 sense, 5′-CAC CGG GAT AAT ATT GAT GGT GTA CGT GTG CTG TCC GTA CAT CAT CAA TAT TGT TCC-3′, and PTEN no. 1 antisense, 5′-AAA AGG AAC AAT ATT GAT GAT GTA CGG ACA GCA CAC GTA CAC CAT CAA TAT TAT CCC-3′; PTEN no. 2 sense, 5′-CAC CGA GTG GGT TTG AAA TAT TAA CGT GTG CTG TCC GTT AAT GTT TCA AGC CCA TTC-3′, and PTEN no. 2 antisense, 5′-AAA AGA ATG GGC TTG AAA CAT TAA CGG ACA GCA CAC GTT AAT ATT TCA AAC CCA CTC-3′; PTEN no. 3 sense, 5′-CAC CGA TTT AGG CTT GAC TTA TAA CGT GTG CTG TCC GTT ATA GGT CAA GTC TAA GTC-3′, and PTEN no. 3 antisense, 5′-AAA AGA CTT AGA CTT GAC CTA TAA CGG ACA GCA CAC GTT ATA AGT CAA GCC TAA ATC-3′; and PTEN no. 4 sense, 5′-CAC CGG GCT AGA GGA AAC TTC ATA CGT GTG CTG TCC GTA TGA GGT TTC CTC TGG TCC-3′, and antisense, 5′-AAA AGG ACC AGA GGA AAC CTC ATA CGG ACA GCA CAC GTA TGA AGT TTC CTC TAG CCC-3′.

Packaging vector (9 μg) (ViraPower packaging mix; Invitrogen Life Technologies), pLenti6/Block-iT with PTEN shRNA or control LacZ shRNA, or GFP expressing pNUTS vector (6 μg), were cotransfected into 293FT packaging cells with Lipofectamine 2000 (35 μl) (Invitrogen Life Technologies). After 48 h the culture supernatants were centrifuged to pellet the released virus and resuspended in 5 ml of StemPro medium. Transduction of human mast cells (HuMCs) or human mast cell line 1 (HMC-1) cells (5×10^6) was conducted by resuspending the cells in the 5 ml of virus containing StemPro medium. Two days after infection, the medium was changed to virus-free Stem Pro, and antibiotic selection (2 μg/ml blasticidin) was initiated following an additional 2-day recovery. After 3 wk of selection, cells were analyzed for FcεRI expression. Cultures were used when >95% of the cells expressed FcεRI.

Cell cultures, activation, lysates, and immunoblots.

The HMC-1 mast cell line was cultured as described (11). HuMCs were developed from $CD34^+$ cells as described (9). Experiments were conducted 8–10 wk after the initiation of HuMC cultures (99% mast cells). FcεRI stimulation of HuMCs (sensitized with biotinylated IgE) was accomplished with the indicated concentration of SA. Lysates were prepared and proteins identified as described (12).

Measurement of cytosolic calcium, degranulation, cytokine production, PIP_3, and apoptosis

Calcium measurements on fura-2 loaded HuMCs were previously described (13). Release (degranulation) of the granule marker β-hexosaminidase was assayed as previously described (9). For cytokine secretion, the human cytokine array ELISA kit from Novagen was used (13). PIP_3 isolation and measurements were done as described (3). For initiation of apoptosis, cells were in StemPro medium without IL-6 and stem cell factor for up to 48 h. Detection of changes in mitochondrial membrane potential and DNA compaction was by flow cytometric measurement with tetramethyl rhodamine methyl ester and DNA compaction with ToPro-3, respectively.

Results and Discussion

Lentiviral-mediated expression of genes (GFP) in HMC-1 cells or $CD34^+$-derived cultured HuMCs was highly successful (Fig. 1). The efficiency of the transduction ranged from 70 to 99% for transient expression of GFP in HMC-1 (Fig. 1*B*) or HuMC (Fig. 1*C*). Selection with blasticidin led to stable protein expression or protein down-regulation (with shRNA) for up to 6 wk (Figs. 1 and 2, and data not shown). Four shRNA sequences were chosen for PTEN down-regulation based on a previously described algorithm (14). All four sequences inhibited PTEN protein expression in HMC-1 cells (Fig. 2*A*). The PI3K-dependent kinase 1 (PDK1)-dependent phosphorylation of Akt (T308) was used as a surrogate measure of increased PIP_3 production following PTEN down-regulation (PDK1 is a PIP_3-binding protein). Constitutive phosphorylation of Akt

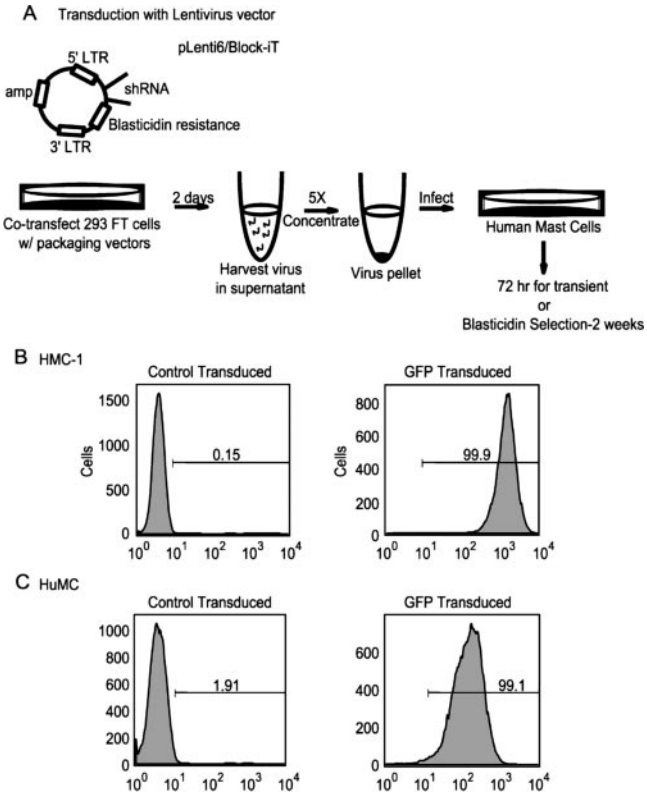

FIGURE 1. Lentivirus-mediated transduction of HuMCs is highly efficient. *A*, Packaging vectors (9 μg) (ViraPower packaging mix; Invitrogen Life Technologies) and pLenti6/Block-iT/PTEN or lacZ shRNA (6 μg) were transfected to 293FT cells. Alternatively, the pNUTS vector carrying a GFP expression cassette was used to assess transduction efficiency. The virus produced was concentrated 5-fold and used to infect 21-day-old cultures of HuMCs. LTR, long terminal repeat; amp, ampicillin-resistance gene. *B* and *C*, Histograms show transient expression of GFP (pNUTS vector) in HMC-1 and HuMCs 4 days after infection. One experiment representative of >10 is shown.

(T308) was increased 3- to 7-fold relative to nontransduced or control lentiviral LacZ shRNA-transduced HMC-1 cells. Direct assessment of PIP_3 production, as shown in Fig. 2*B*, revealed a constitutive increase on the average of 1.5- to 2-fold. Because transduction with shRNA no. 1 resulted in effective loss of PTEN protein (Fig. 2*A*) and in a constitutive increase of at least 2-fold in PIP_3 levels (Fig. 2*B*), this shRNA sequence was used to analyze the role of PTEN in HuMC.

PTEN-deficiency also caused a constitutive phosphorylation of Akt (T308) in HuMCs (Fig. 3*A*). No significant increase in PIP_3 or Akt phosphorylation was observed in control wild-type cells (Figs. 2 and 3), arguing that control of these responses depends on the constitutive activity of PTEN. This differs from SHIP-null murine bone marrow-derived mast cells, where resting cells showed minimal phosphorylation of Akt (5).

After FcεRI-stimulation, Akt (T308) phosphorylation in PTEN-deficient cells was further increased. Akt is well known to function as a PI3K-dependent prosurvival protein through its regulation of Bcl family members, the Forkhead family of transcription factors, and p53 family members (15). Analysis of events that can initiate apoptosis (mitochondrial membrane potential and DNA compaction) under conditions of growth factor starvation revealed a significant reduction (16 h) in these events in PTEN-deficient cells (Fig. 3*B*). Thymidine incorporation studies showed a slight increase (1.5-fold) in the rate of

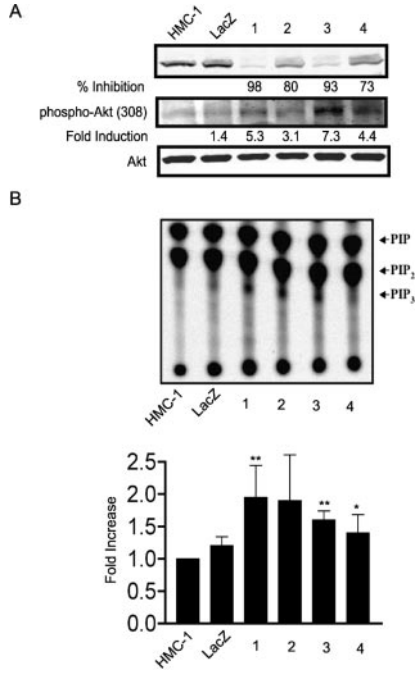

FIGURE 2. Lentiviral shRNA silencing of PTEN increases PIP_3 production and Akt phosphorylation. *A*, Nontransduced HMC-1 cells or cells transduced with LacZ shRNA or four lentiviral shRNA constructs (1–4) were assessed for loss of PTEN and for Akt (T308) phosphorylation (phospho-Akt). PTEN loss is shown as the percentage (%) of inhibition, and fold induction of Akt (T308) phosphorylation is also indicated. *B*, PIP_3 production is enhanced by PTEN down-regulation with four lentiviral shRNA constructs (1–4). Quantitation of all experiments is shown in the h (*, $p < 0.05$; **, $p < 0.01$).

proliferation of PTEN-deficient cells (data not shown). Prolonged growth factor starvation (>48 h), however, caused the death of all cells.

The effect of PTEN-deficiency on FcεRI-initiated signaling was explored. The phosphorylation of Src family kinases in the activation loop tyrosine (Y416) was not detected in resting cells but was identically stimulated in control or PTEN-deficient HuMCs (Fig. 4*A*). LAT (linker for activation of T cells) phosphorylation at Y191 contributed to the stability of this signaling complex (16), required FcεRI stimulation, and was independent of PTEN expression. Phosphorylation of phospholipase Cγ, which binds to LAT and hydrolyzes PIP_2, generating inositol 1,4,5-trisphosphate to cause calcium mobilization, was significantly increased (~1.5-fold at 45 s; $p \leq 0.05$) in PTEN-deficient cells (Fig. 4*A*). This was linked to enhanced FcεRI-dependent calcium mobilization in PTEN-deficient HuMCs (Fig. 4*B*) and increased degranulation as compared with control cells (Fig. 4*C*). No differences were observed in basal (spontaneous) degranulation. The results demonstrate that the loss of PTEN is insufficient to initiate a constitutive degranulation response, but the increased phospholipase Cγ (PLCγ) activation and calcium responses seemingly served to enhance FcεRI-stimulated secretion.

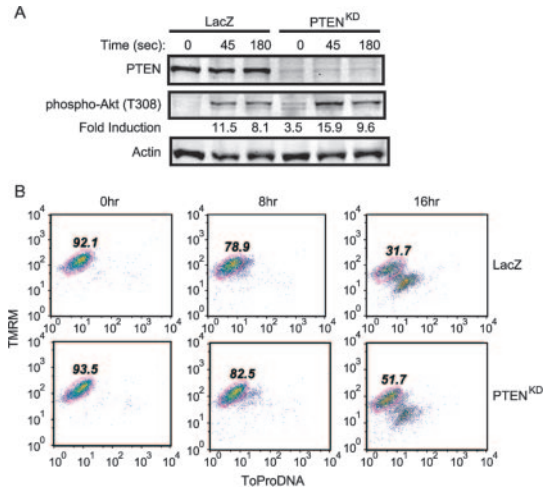

FIGURE 3. PTEN-deficient HuMCs have increased Akt phosphorylation and decreased signals for initiation of apoptosis. *A*, Akt is constitutively phosphorylated (phospho-Akt) in PTEN-deficient HuMCs, and FcεRI stimulation further enhances this event. *B*, IL-6 and the stem cell factor were withdrawn from human mast cell cultures for the indicated times. Initiation of apoptosis was determined using the mitochondrial dye tetramethyl rhodamine methyl ester (TMRM) and the DNA stain ToPro-3 (ToProDNA). The percentage of live/nonapoptotic cells is indicated (red gate). One representative experiment of four is shown.

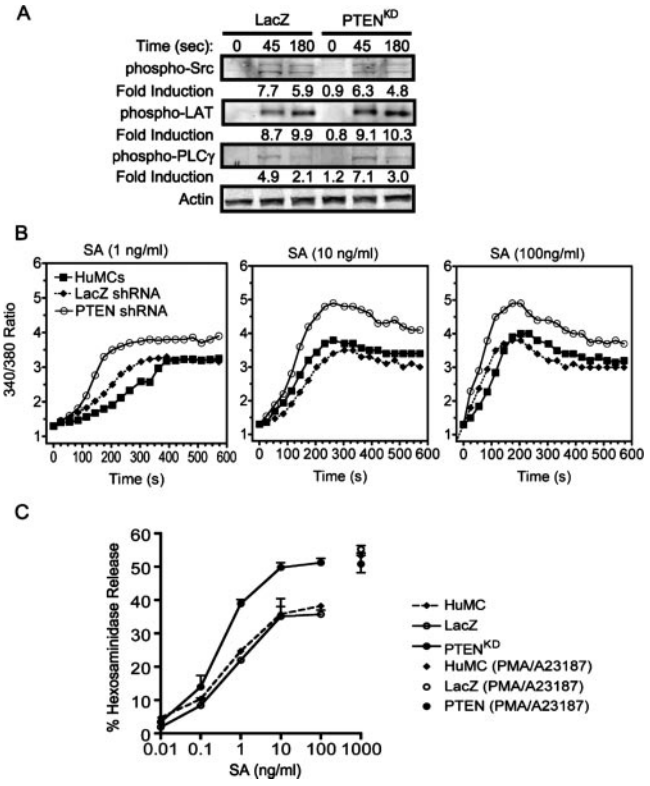

FIGURE 4. PTEN-deficient HuMCs showed increased phosphorylation of PLCγ, calcium mobilization, and degranulation. *A*, Biotinylated IgE-sensitized HuMCs were stimulated or not stimulated with 100 ng/ml SA. The phosphorylation (phospho-) of PLCγ (Y783), LAT (Y191), and Src (Y416) was determined. Actin was used for normalization of protein loading. One representative experiments of three is shown. *B*, fura-2 loaded HuMCs (sensitized as in *A*) were stimulated as indicated to measure intracellular-free Ca^{2+}. The ratio of fluorescence emission at 510 nm when cells are excited at 340 and 380 nm is shown. Data are representative of three experiments using duplicate samples from individual HuMC cultures. The calcium response of HuMC or LacZ controls was not significantly different. *C*, Degranulation from indicated cells was measured by β-hexosaminidase release of IgE-sensitized cells stimulated with 0.01–100 ng/ml SA for 30 min. Maximal degranulation was determined by PMA (20 nM) and the calcium ionophore A23187 (200 nM) stimulation. Degranulation is expressed as the percentage of total intracellular β-hexosaminidase released to the medium. Data are means ± SEM from six individual experiments.

The importance of PI3K activity for the de novo synthesis of cytokines in mast cells has been well demonstrated (2, 4); however, the signals influenced by PIP_3 are unclear. MAP kinases (ERK, JNK, and p38) phosphorylate transcription factors known to regulate cytokine gene expression (17). Fig. 5*A* shows that PTEN deficiency caused a minimal constitutive phosphorylation of ERK, whereas constitutive phosphorylation of JNK and p38 reached 60–70% of the stimulated response. Because MAP kinases are linked to transcription factor activation, we analyzed the phosphorylation of IκB kinase (IKK), activating transcription factor 2 (ATF2), and c-Jun. IKK and c-Jun phosphorylation was not significantly altered in PTEN-deficient cells (Fig. 5*B*); however, constitutive ATF2 phosphorylation was observed.

Strikingly, both IL-8 and GM-CSF were found to be constitutively secreted from PTEN-deficient cells (Fig. 5*C*), and ATF activity has been associated with the transcription of both of these genes (18, 19). The level of IL-8 secretion was similar to that of FcεRI-stimulated control cells, whereas GM-CSF secretion was ~50% of the stimulated response. Thus, these genes appear to be highly dependent on PIP_3 production for their activation. This possibility was further explored by treatment of control or PTEN-deficient cells with the PI3K inhibitor wortmannin. Cytokine secretion from control cells was inhibited (ranging from 60 to 90%). However, wortmannin failed to block the constitutive or stimulated secretion of cytokines from the PTEN-deficient HuMCs. Thus, PTEN deficiency bypassed the need for FcεRI-stimulated PI3K activity.

SHIP-1 and −2 are present in PTEN-deficient cells, advancing the view that PTEN is key in the homeostatic control of PIP_3 levels. This hypothesis is consistent with the tumor suppressive properties of PTEN (7). Control of both Akt and MAP kinase basal activity by PTEN appears to be a vital function that governs the constitutive production of proinflammatory cytokines linked to tumor promotion (20). In FcεRI-initiated responses, PTEN appears to be contributory for PIP_3 regulation. However, the FcεRI-stimulated phenotype of SHIP-null mast cells is almost identical with that of FcεRI-stimulated PTEN-deficient mast cells (5), suggesting significant redundancy in the regulation of PIP_3 levels in stimulated cells. Of particular interest is whether PTEN function in homeostasis is entirely independent of cell stimulation (as suggested herein) and whether loss/decline of PTEN activity might alter mast cell function in health and disease.

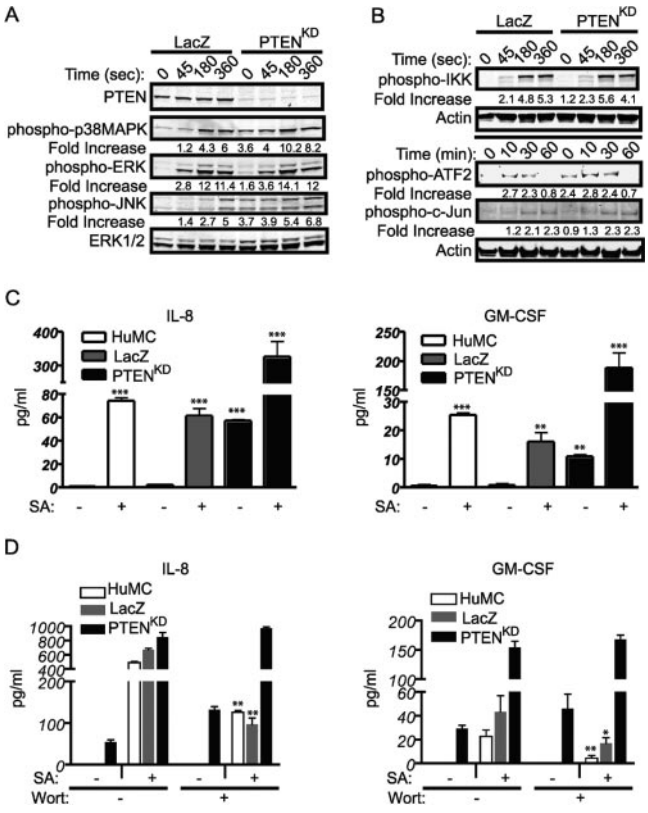

FIGURE 5. Constitutive and enhanced FcεRI-stimulated MAP kinase phosphorylation and cytokine secretion in PTEN-deficient human mast cells. *A*, Biotinylated IgE-sensitized HuMCs were stimulated or not stimulated with 100 ng/ml SA. The PTEN expression level and the phosphorylation of MAP kinases was determined with Abs to PTEN and to phosphorylated (phospho-) p38, ERK, and JNK. The ERK protein was used for normalization. Fold increase is normalized to nonstimulated LacZ control. *B*, PTEN-deficient HuMCs were stimulated and processed as described above for the indicated times. Phosphorylation of IKK, c-Jun, and ATF2 was detected by immunoblot with phosphorylated (phospho-) IKK, c-Jun, and ATF2 antibodies. Actin protein was used for loading control. One representative experiment of three is shown (*A* and *B*). *C*, PTEN-deficient HuMCs were stimulated as previously described (13). Secreted IL-8 and GM-CSF were measured by ELISA. Data are reported as mean ± SEM of three individual experiments. Significance is relative to wild-type cells (**, $p < 0.005$; ***, $p < 0.0005$). *D*, Experiments were conducted as in *C*, but 30 nM wortmannin (Wort., a PI3K inhibitor) was added to nonstimulated or FcεRI-stimulated cells for 60 min before stimulation. Cytokine secretion was measured as in *C*. Significance is relative to untreated cells (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$).

Disclosures

The authors have no financial conflict of interest.

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