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PTEN Negatively Regulates Engulfment of Apoptotic Cells by Modulating Activation of Rac GTPase

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Efficient clearance of apoptotic cells by phagocytes (efferocytosis) is critical for normal tissue homeostasis and regulation of the immune system. Apoptotic cells are recognized by a vast repertoire of receptors on macrophage that lead to transient formation of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3] and subsequent cytoskeletal reorganization necessary for engulfment. Certain PI3K isoforms are required for engulfment of apoptotic cells, but relatively little is known about the role of lipid phosphatases in this process. In this study, we report that the activity of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a phosphatidylinositol 3-phosphatase, is elevated upon efferocytosis. Depletion of PTEN in macrophage results in elevated PtdIns(3,4,5)P3 production and enhanced phagocytic ability both in vivo and in vitro, whereas overexpression of wild-type PTEN abrogates this process. Loss of PTEN in macrophage leads to activation of the pleckstrin homology domain-containing guanine-nucleotide exchange factor Vav1 and subsequent activation of Rac1 GTPase, resulting in increased amounts of F-actin upon engulfment of apoptotic cells. PTEN disruption also leads to increased production of anti-inflammatory cytokine IL-10 and decreased production of proinflammatory IL-6 and TNF-α upon engulfment of apoptotic cells. These data suggest that PTEN exerts control over efferocytosis potentially by regulating PtdIns(3,4,5)P3 levels that modulate Rac GTPase and F-actin reorganization through Vav1 exchange factor and enhancing apoptotic cell-induced anti-inflammatory response. The Journal of Immunology, 2011, 187: 5783–5794.

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Abbreviations used in this article: CB, cytoskeletal fraction buffer; Gas6, growth-arrest–specific 6; GPCR, G protein-coupled receptor; MFG-E8, milk-fat globule E8; PH, pleckstrin homology; PS, phosphatidylserine; PtdIns(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; WT, wild-type.

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sitol 5-phosphatase, convert PtdIns(3,4,5)P3 to PtdIns(4,5)P2 and PtdIns(3,4)P2, respectively. Relatively little is known about the role of these lipid phosphatases in the regulation of apoptotic cell-mediated signaling. The roles of PtdIns(3,4,5)P3 and PTEN in infection and inflammation have been extensively examined. Blocking the kinase activity of P3K leads to the impaired recruitment of neutrophils to inflammatory sites in vivo (19–26). In contrast, increasing PtdIns(3,4,5)P3 signaling by depleting PTEN enhances cell mobility (27–31) and improves neutrophil recruitment to the inflamed peritoneal cavity (32). The disruption of PTEN also enhances neutrophil function in a bacterial pneumonia model, leading to increased engulfment of bacteria (33, 34). In the current study, we have investigated whether the loss of PTEN can result in macrophage activation, leading to the enhanced clearance of apoptotic cells and faster resolution of inflammation.

In this study, we demonstrate that PTEN negatively regulates efferocytosis. Overexpression of PTEN reduces the engulfment of apoptotic cells, and disruption of PTEN increases the engulfment of apoptotic cells both in vitro and in vivo. We also show that PTEN activity is upregulated during the engulfment of apoptotic cells, although PTEN does not localize to the phagocytic cup like PtdIns (3,4,5)P3. The loss of PTEN in macrophages results in the enhanced formation of PtdIns(3,4,5)P3, leading to activation of the pleckstrin homology (PH) domain-containing exchange factor Vav1, which induces the activation of Rac GTPase and the subsequent polymerization of F-actin and enhances the engulfment of apoptotic cells. Consistent with augmented engulfment, the loss of PTEN increases the production of anti-inflammatory cytokine (IL-10) and decreases the production of proinflammatory (IL-6 and TNF-α) cytokines upon efferocytosis.

Materials and Methods

Mice

The conditional PTEN knockout mouse (PTEnko/koCre) and the myeloid-specific Cre mouse were purchased from The Jackson Laboratory (Bar Harbor, ME). The experimental myeloid-specific PTEN knockout mice were generated as previously described (32). In all the experiments performed using knockout mice (PTEnko/koCre; Cre+ or PTEnko/koCre; Cre−), we included corresponding littermates (PTEnwt/wt; Cre+ or PTEnwt/wt; Cre−) as wild-type controls. Rac1int/−/− (35), Rac2−/− (36), and Rac3−/− (37) mice have been described elsewhere. All procedures involving mice were approved and monitored by the Children’s Hospital Animal Care and Use Committee.

Cells, Abs, plasmids, and reagents

RAW264.7 (American Type Culture Collection) cells were maintained in DMEM containing 10% FCS and transfected with plasmids encoding PTEN-Citrine (38), myristoylated-Akt enhanced GFP (myr-Akt-EGFP), or Akt-PH-EGFP using the Amaxa nucleofection kit according to the manufacturer’s protocol. Mouse peritoneal macrophages were prepared by injecting 1 ml 3% thiglycolate (Sigma) i.p. The peritoneal lavage fluid was collected after 3 d, and the collected cells were cultured in RPMI 1640 containing 10% FCS. Mouse thymocytes were isolated by surgically removing the thymus glands. The glands were rinsed, and the thymocytes were resuspended in 10 ml RPMI 1640 containing 10% FCS by smashing the glands with the flat plastic head of a 10-ml syringe plunger. Murine bone marrow neutrophils were isolated using the neutrophil enrichment kit from Stem Cell Technologies according to the manufacturer’s protocol. Abs to PTEN (Ser-380/Thr-382/Thr-383), total-PTEN, phospho-Akt, phospho-ERK1/2, phospho-Pyk2, and actin were obtained from Cell Signaling Technologies. The Abs to Vav1 and Rac1 were obtained from Santa Cruz Biotechnology, and the anti-phospho-Tyr Ab was from Millipore. TGX221 and AS225242 were obtained from Cayman Chemicals. Compound 15e was from Santa Cruz Biotechnology. The Akt-VIII was from EMD Biosciences, and the Wortmannin was from Tocris Biosciences.

In vitro phagocytosis of apoptotic cells

RAW264.7 macrophages or thiglycolate-elicted peritoneal macrophages were incubated with apoptotic neutrophils or thymocytes for 90 min. The unengulfed cells were removed by washing with PBS, and the phagocytosis of apoptotic cells by macrophages was detected by staining with HEMA3 (Fisher) following the manufacturer’s guidelines. The percentage of macrophages containing at least one apoptotic body was quantified to determine the percent efferocytosis, and the number of apoptotic bodies per macrophage was determined to calculate the efferocytic index. Apoptotic neutrophils were obtained by culturing neutrophils in RPMI 1640 for 1 d, which typically resulted in 30–40% apoptotic cells. Thymocyte apoptosis was induced by incubation in 1 mM dexamethasone overnight, which generated >85% apoptotic cells.

In vivo efferocytosis

In the peritonitis model, wild-type and PTEN−/− mice were injected i.p. with 3% thiglycolate to induce inflammation. At day 3, when the number of peritoneal macrophages was maximal, 40 × 106 apoptotic thymocytes resuspended in PBS were injected into the peritoneum. After 90 min, the cells from the peritoneal lavage were analyzed for the percentage of macrophages containing engulfed apoptotic cells. The dexamethasone-induced thymic involution was performed as described previously with slight modifications (2). To induce thymic involution caused by thymocyte apoptosis, 250 µg dexamethasone was injected i.p. into mice for 4 or 8 h. Treatment with PBS in an equivalent amount of DMSO (solvent) for 8 h was used as the control. The mice were sacrificed, the thymuses were isolated, and the total number of cells were counted. The total numbers of annexin V+ apoptotic cells and Mac1+ macrophages were determined by FACS.

PTEN activity assay

Thiglycolate-elicited peritoneal macrophages were treated with MFG-E8 (1 µg/ml) or Gas6 (1 µg/ml) or incubated with apoptotic thymocytes for the indicated time. The unengulfed apoptotic cells were washed with PBS three times. The macrophages were then lysed using RIPA buffer containing 150 mM NaCl, 1.0% IPEGAL, 0.1% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8 (Sigma). The cell lysates were incubated overnight at 4°C with protein-A Sepharose beads coated with anti-PTEN (Ser-380/Thr-382/Thr-383) Ab (Cell Signaling Technologies). The beads were washed three times with the PTEN reaction buffer containing 25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2.7 mM KCl, and 10 mM DTT. The PTEN pulled down was incubated with 5000 pmol water-soluble PtdIns(3,4,5)P3 substrate (Echelon Biosciences) for 30–60 min at 37°C. The free phosphate generated through PTEN activity was then quantified using the malachite green phosphatase assay kit according to the manufacturer’s protocol (Echelon Biosciences). The percent of PtdIns(3,4,5)P3 conversion was determined at each time point as: [(free phosphate in test reaction, pmol) – (free phosphate in background, pmol)] × 100%/5000 pmol. The free phosphate in the background was the amount of phosphate in the “substrate only [PtdIns(3,4,5)P3]” controls. The cell lysates were also analyzed for the levels of total PTEN.

ELISA

Peritoneal macrophages (0.5 × 106) from wild-type (WT) or PTEN−/− mice were plated in a 24-well plate and cultured for 12–16 h in RPMI 1640 containing 10% FCS at 37°C. The cells were then treated with LPS in the presence or absence of apoptotic cells (5 × 105 per well) for 16 h. The levels of the proinflammatory cytokines IL-6 and TNF-α and the anti-inflammatory cytokine IL-10 in the culture supernatants were determined by ELISA (eBioscience) according to the manufacturer’s guidelines.

Rac and Vav1 activation assays

RAW264.7 or peritoneal macrophages were treated with apoptotic cells for the indicated time periods, and the unengulfed cells were washed off as previously described. For the Vav1 activation assay, cells were lysed in lysis buffer containing 20 mM Tris-HCl, pH 8, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 2 mM EDTA and supplemented with protease inhibitor mixture (Roche). The cell lysates were incubated with Protein A/G Plus Sepharose beads coated with Vav1 Ab (Santa Cruz Biotechnology) for 1 h at 4°C. The immunoprecipitates were washed with lysis buffer, resolved by SDS-PAGE, and analyzed for levels of Vav1 and phospho-Tyr (clone 4G10; Millipore). For the Rac activation assay, the cells were lysed in lysis buffer containing 50 mM Tris pH 7.5, 10 mM MgCl2, 300 mM NaCl, and 1% Triton X-100 supplemented with protease inhibitors (Roche). Portions of the cell lysates were saved for input controls, and 500 µg total protein from each sample was then incubated with glutathione Sepharose beads coated with GST-PK-PBD to pull down active Rac. The pulldown eluates were washed in wash buffer containing 25 mM Tris pH 7.5, 30 mM MgCl2, and 40 mM NaCl, resolved by SDS-PAGE, and immunoblotted with Rac1.
myr-Akt. Results shown are means ± SD (n > 600). *p < 0.005.

FIGURE 1. PI3K but not Akt is required for engulfment of apoptotic cell by macrophage. A. Effect of isoform-specific PI3K inhibitors on engulfment of apoptotic cell by macrophages. Macrophages pretreated with 50 nM wortmannin (pan-specific), 50 nM Compound 15e (p110α-specific), 50 nM TGX-221 (p110β-specific), or 50 nM AS252424 (p110γ-specific) were incubated with apoptotic thymocytes for 90 min, and the engulfment of apoptotic cells was analyzed by HEM3 staining. Percentage of macrophages containing one or more apoptotic body is shown. B. Transient formation of PtdIns(3,4,5)P3 products in the process. Compound 15e, which targets p110α, and TGX221, which targets p110β, also showed significant inhibitory effects, indicating that class IA PI3Ks are important for efferocytosis. In contrast, AS252424, which targets p110γ, did not have any effect on efferocytosis, indicating that the p110γ [the major G protein-coupled receptor (GPCR)-activated PI3K isoform] mediated formation of PtdIns(3,4,5)P3 is not involved in the process (Fig. 1A). As a control for the efficacy of AS252424 in inhibiting p110γ-mediated PI3K–Akt signaling, we treated macrophages with fMLF (a GPCR ligand) in the presence or absence of 50 nM AS252424 for 30 min. The cell lysates were analyzed for Akt phosphorylation. The addition of AS252424 completely inhibited Akt phosphorylation in macrophages upon stimulation with fMLF.
fMLF, indicating the inhibition of p110γ by AS252424 (Supplemental Fig. 1A).

Next, we examined the localization of PtdIns(3,4,5)P3 during efferocytosis. We transfected RAW264.7 macrophage-like cells with an Akt-PH-EGFP construct. One day after the transfection, RAW264.7 cells were coincubated with CMTMR-labeled apoptotic thymocytes for 1 h. Akt-PH-EGFP was enriched at the phagocytic cup during efferocytosis, indicating that PtdIns(3,4,5)P3 was formed at this location (Fig. 1A, top panel). The enrichment of PtdIns(3,4,5)P3 at the phagocytic cup was transient. It returned to basal level upon the completion of engulfment, suggesting that PtdIns(3,4,5)P3 may play a role in the engulfment of apoptotic cells by macrophages (Fig. 1A, bottom panel).

The serine/threonine kinase Akt is one of the most well-studied PtdIns(3,4,5)P3 effector molecules. The binding of Akt to PtdIns(3,4,5)P3 through its PH domain facilitates its membrane localization and activation, which subsequently regulates diverse cellular processes like cell proliferation, cell cycle, and cell migration. To test whether Akt influences the process of efferocytosis, we first used a pharmacological inhibitor of Akt, Akti-VIII. Treatment of macrophages with Akti-VIII for 30 min completely blocked Akt phosphorylation and thus its activation stimulation with fMLF (Supplemental Fig. 1B). Peritoneal macrophages were either pretreated with 50 nM wortmannin or 7.6 μM Akti or left untreated, then fed with apoptotic thymocytes for 90 min. Treatment with wortmannin severely reduced efferocytosis, but treatment with Akti had no significant effect on efferocytosis (Fig. 1C). To investigate further the role of Akt in efferocytosis, we transfected RAW264.7 cells with a constitutively activated form of Akt, myr-Akt, in which Akt is myristoylated and is always in the plasma membrane. As expected, transfection with myr-Akt resulted in signaling in the cell, as evidenced by the increased levels of phosphorylated Akt (phospho-Akt), which serves as a marker for PtdIns(3,4,5)P3 formation. In addition, PTEN disruption resulted in increased levels of activated Akt in macrophages stimulated with fMLF or PMA (Fig. 1A). In addition, Akt phosphorylation induced by efferocytosis bridging proteins MFG-E8 and Gas6, which elicit downstream signaling through α5β1-integrin and receptor tyrosine kinase Mer, respectively, was also elevated in PTEN+/− macrophages (Fig. 3B). To determine whether PTEN plays a role in efferocytosis, we coincubated PTEN-null peritoneal macrophages with either dexamethasone-induced apoptotic thymocytes or apoptotic neutrophils for 90 min. The efficiency of efferocytosis (percent of macrophage containing one or more apoptotic body) and the phagocytic index (number of apoptotic bodies per macrophage) were analyzed. The PTEN+/− macrophages consistently engulfed increased quantities of apoptotic neutrophils or thymocytes. WT macrophages had a phagocytic index of ∼1, and PTEN−/− macrophages had a phagocytic index of 1.6 (Fig. 3C). About 15% of the WT macrophages engulfed apoptotic neutrophils, whereas 23% of PTEN+/− macrophages engulfed apoptotic neutrophils. Likewise, ~50% of WT macrophages engulfed apoptotic thymocytes, whereas >65% of PTEN+/− macrophages engulfed apoptotic thymocytes. Viable cells were not engulfed by either WT or PTEN−/− macrophages (Fig. 3D, 3E), and WT and PTEN−/− macrophages exhibited similar binding to apoptotic cells (Supplemental Fig. 3). Next, we compared the kinetics of apoptotic cell engulfment in WT and PTEN−/− macrophages. WT and PTEN−/− macrophages were fed with apoptotic cells for 0, 15, 30, 60, or 90 min, and the percentages of macrophages that engulfed apoptotic cells were assessed. At all of the time points, we observed that PTEN+/− macrophages engulfed more apoptotic cells, but the difference in engulfment of apoptotic cells was larger at the relatively earlier time points such as 15 and 30 min (Fig. 3F).

Notably, the kinetics of apoptotic cell engulfment in WT was similar to the kinetics of PTEN upregulation observed during efferocytosis.

Disruption of PTEN enhances engulfment of apoptotic cells by macrophages

The complete loss of PTEN in mice leads to embryonic lethality. To examine the role of PTEN and PtdIns(3,4,5)P3 signaling in efferocytosis, we used a conditional myeloid-specific PTEN knockout mouse generated by crossing a PTEN-floxed mouse with a line expressing myeloid-specific Cre. In this Cre line, the expression of the Cre recombinase gene is under the control of the lysozyme promoter, which is expressed only in cells of the myeloid lineage, including monocytes, mature macrophages, and neutrophils. Western blot analysis indicated that the expression of PTEN protein was completely abolished in thioglycolate-elicited peritoneal macrophages isolated from either PTENfl/fl;Cre+/− or PTENfl/fl;Cre+/− mice (PTEN−/− macrophages) (Fig. 3A). The loss of PTEN resulted in elevated basal PtdIns(3,4,5)P3 signaling in the cell, as evidenced by the increased levels of phosphorylated Akt (phospho-Akt), which serves as a marker for PtdIns(3,4,5)P3 formation. In addition, PTEN disruption resulted in increased levels of activated Akt in macrophages stimulated with fMLF or PMA (Fig. 3A). In addition, Akt phosphorylation induced by efferocytosis bridging proteins MFG-E8 and Gas6, which elicit downstream signaling through α5β1-integrin and receptor tyrosine kinase Mer, respectively, was also elevated in PTEN−/− macrophages (Fig. 3B). To determine whether PTEN plays a role in efferocytosis, we coincubated PTEN-null peritoneal macrophages with either dexamethasone-induced apoptotic thymocytes or apoptotic neutrophils for 90 min. The efficiency of efferocytosis (percent of macrophage containing one or more apoptotic body) and the phagocytic index (number of apoptotic bodies per macrophage) were analyzed. The PTEN−/− macrophages consistently engulfed increased quantities of apoptotic neutrophils or thymocytes. WT macrophages had a phagocytic index of ∼1, and PTEN−/− macrophages had a phagocytic index of 1.6 (Fig. 3C). About 15% of the WT macrophages engulfed apoptotic neutrophils, whereas 23% of PTEN+/− macrophages engulfed apoptotic neutrophils. Likewise, ~50% of WT macrophages engulfed apoptotic thymocytes, whereas >65% of PTEN+/− macrophages engulfed apoptotic thymocytes. Viable cells were not engulfed by either WT or PTEN−/− macrophages (Fig. 3D, 3E), and WT and PTEN−/− macrophages exhibited similar binding to apoptotic cells (Supplemental Fig. 3). Next, we compared the kinetics of apoptotic cell engulfment in WT and PTEN−/− macrophages. WT and PTEN−/− macrophages were fed with apoptotic cells for 0, 15, 30, 60, or 90 min, and the percentages of macrophages that engulfed apoptotic cells were assessed. At all of the time points, we observed that PTEN+/− macrophages engulfed more apoptotic cells, but the difference in engulfment of apoptotic cells was larger at the relatively earlier time points such as 15 and 30 min (Fig. 3F). Notably, the kinetics of apoptotic cell engulfment in WT was similar to the kinetics of PTEN upregulation observed during efferocytosis.
It is possible that PTEN activity was upregulated during efferocytosis and remained elevated for a prolonged time to maintain the low PtdIns(3,4,5)P3 in the cells that have finished engulfment. We next tested if the overexpression of PTEN, which reduces the cellular levels of PtdIns(3,4,5)P3, could affect efferocytosis. Peritoneal macrophages were incubated with apoptotic thymocytes for the indicated time and washed to remove unadhered thymocytes. Cells were then lysed, and PTEN was immunoprecipitated. PTEN activity was assessed by its ability to convert PtdIns(3,4,5)P3 to PtdIns(4,5)P2 and free phosphate. Free phosphates generated were measured by a colorimetric assay using malachite green. Data are representative of three independent experiments done in triplicate. Results shown are means ± SD. *p < 0.05.

Disruption of PTEN enhances clearance of apoptotic cells in vivo

To determine whether the loss of PTEN in macrophages had any effect on the clearance of apoptotic cells in vivo, we used the following two in vivo models: the clearance of apoptotic cells in thioglycolate-elicited peritonitis and the clearance of dexamethasone-induced apoptotic thymocytes in the thymus. Thioglycolate is a polysaccharide mixture that has been widely used to induce mild inflammation, thus providing information about events that occur during inflammation. In the peritonitis model, WT and PTEN−/− mice were treated i.p. with 3% thioglycolate to induce inflammation. After 3 d, macrophages represented the major cell type in the peritoneal cavity. Apoptotic thymocytes (40 × 10⁶) were injected into the peritoneum and incubated for 90 min. The peritoneal cells were flushed out and analyzed by HEMA3 staining. We observed engulfment of apoptotic thymocytes by perito-
neal macrophages in both the WT and PTEN−/− mice, but significantly more macrophages from the PTEN−/− mice had engulfed apoptotic cells (∼26%) compared with WT macrophages that had engulfed apoptotic cells (∼18%) (Fig. 4A).

In the second model, dexamethasone was used to induce thymic involution. Dexamethasone is taken up rapidly by T cells and induces apoptosis, leading to shrinkage in the size of the thymus and a reduction in cell number. The apoptotic cells are cleared by macrophages present in the thymus. In our study, dexamethasone was injected into WT or PTEN−/− mice, and efferocytosis was assessed 4 and 8 h after the injection. The thymuses were extracted from the mice, and the weights and numbers of thymocytes were analyzed. Upon dexamethasone treatment, the thymuses from both WT and PTEN−/− mice exhibited reduced size and weight and manifested decreased total thymocyte numbers. However, 8 h after dexamethasone treatment, the thymuses isolated from PTEN−/− mice weighed less (Fig. 4B) and contained fewer thymocytes than those from WT mice (Fig. 4C). When the number of apoptotic thymocytes was analyzed, we found that in WT mice, the number of apoptotic cells per thymus...
gradually increased in the first 8 h. In contrast, in PTEN$^{-/-}$ mice, there was an increase in the number of apoptotic cells upon treatment with dexamethasone for the first 4 h, followed by a reduction in apoptotic cells in the thymus by 8 h (Fig. 4D). Because the numbers of macrophages in the thymus were similar in the WT and PTEN$^{-/-}$ mice (Fig. 4E), the reduced levels of apoptotic thymocytes in the PTEN$^{-/-}$ mice was likely due to enhanced clearance by macrophages in these mice.

Alteration of cytokine production by PTEN$^{-/-}$ macrophages in response to engulfment of apoptotic cells

It has been established that the phagocytosis of apoptotic cells suppresses the autoimmune response by releasing immunosuppressive cytokines, such as TGF-β and IL-10, and inhibiting the production of proinflammatory cytokines, such as IL-6, TNF-α, IL-12, and IL-1β. As the deletion of PTEN led to more efficient clearance of apoptotic cells through the upregulation of PtdIns(3,4,5)P3 signaling, we wondered whether the loss of PTEN would also affect cytokine production upon efferocytosis. We stimulated thioglycolate-elicited WT and PTEN$^{-/-}$ macrophages with LPS in the presence or absence of apoptotic cells. The cytokines secreted in response to LPS and efferocytosis were analyzed by ELISA. As expected, incubation with apoptotic cells significantly augmented LPS-induced secretion of the anti-inflammatory cytokine IL-10 (Fig. 5A) and reduced the secretion of the inflammatory cytokines IL-6 (Fig. 5B) and TNF-α (Fig. 5C) in both WT and PTEN$^{-/-}$ macrophages. However, in PTEN$^{-/-}$ macrophages, the levels of the proinflammatory cytokines IL-6 and TNF-α were much lower, and the production of the anti-inflammatory cytokine IL-10 was significantly enhanced in the presence of apoptotic cells.

Notably, upon treatment with LPS alone, the levels of IL-10 secreted by PTEN$^{-/-}$ macrophages were also significantly higher than those secreted by WT macrophages, whereas the levels of the proinflammatory cytokines IL-6 and TNF-α were similar in WT and PTEN$^{-/-}$ macrophages. The PtdIns(3,4,5)P3–Akt signaling pathway has been implicated in the regulation of
both proinflammatory and anti-inflammatory cytokines. It has been demonstrated that TLR-induced PI3K–Akt activation and the subsequent phosphorylation and inactivation of GSK-3β inhibits the NF-κB–driven proinflammatory response (42–47) and enhances the expression of the immunosuppressive cytokine IL-10 (48). Accordingly, we analyzed the levels of phosphorylated GSK-3β (Ser-9) in WT and PTEN−/− macrophages and found that the levels of phospho–GSK-3β (inactive form) in PTEN−/− macrophages were drastically elevated compared with those in WT macrophages (Fig. 5D). Thus, the augmentation of LPS-induced IL-10 production could simply be a result of enhanced GSK-3β phosphorylation and inactivation induced by PTEN disruption. However, it appeared that PTEN disruption-induced elevation of PtdIns(3,4,5)P3 signaling was not sufficient to alter the production of inflammatory cytokines (IL-6 and TNF-α) in our experimental system (Fig. 5B, 5C).

Enhanced Rac activation in PTEN−/− macrophages

Rac subfamily GTPases have been implicated in the process of phagocytosis and have been shown to be key regulators of apoptotic cell engulfment. We next examined the activation of Rac GTPase in WT and PTEN−/− macrophages using GST-PAK-PBD to pull down activated Rac. Upon engulfment of apoptotic cells, an increase in Rac activation was observed. Both under basal conditions and when stimulated with apoptotic cells for 30 min, PTEN−/− macrophages had much higher levels of Rac1-GTP (Fig. 6A). We next examined the roles of different Rac isoforms in efferocytosis. The mouse genome encodes three Rac isoforms: Rac 1, 2, and 3. Rac1 and Rac2 compose 99% of the Rac in macrophages, and Rac3 is a minor isoform (49, 50). We used mice deficient in the three Rac isoforms to test their roles in efferocytosis. The mouse genome encodes three Rac isoforms: Rac1, 2, and 3. Rac1 and Rac2 compose 99% of the Rac in macrophages, and Rac3 is a minor isoform (49, 50). We used mice deficient in the three Rac isoforms to test their roles in efferocytosis. The mouse genome encodes three Rac isoforms: Rac1, 2, and 3. Rac1 and Rac2 compose 99% of the Rac in macrophages, and Rac3 is a minor isoform (49, 50). We used mice deficient in the three Rac isoforms to test their roles in efferocytosis. To investigate the mechanism by which Rac activity is elevated in PTEN−/− macrophages, we investigated the involvement of other PH domain-containing Rac guanine-nucleotide exchange factors in the regulation of efferocytosis. Vav-family guanine exchange factors are PH domain-containing proteins that are recruited to the membrane upon PtdIns(3,4,5)P3 formation and activated by tyrosine phosphorylation by protein tyrosine kinases. Vav-family GTPases act as exchange factors for Rac and cdc42 GTPases, which regulate actin polymerization. The engulfment of apoptotic cells requires the activation of Rac GTPase and subsequent actin polymerization to form the phagocytic cup. Immunoprecipitation analysis revealed that PTEN−/− macrophages had much higher levels of phosphorylated (activated) Vav1 compared with those of WT macrophages (Fig. 7A), indicating that in PTEN−/− macrophages, the increased Vav1 activation may result in the enhanced Rac1 activation and augmented actin reorganization required for more efficient engulfment of apoptotic cells. We next examined whether Vav1 is activated by phosphorylation upon efferocytosis.
Immunoprecipitation analysis showed that treatment with apoptotic cells resulted in gradually increased levels of Vav1 phosphorylation, which reached a maximum at 30 min (Fig. 7B). These results indicate that Vav1 is activated in macrophages upon the engulfment of apoptotic cells. Because the levels of Vav1 protein were undetectable or extremely low in apoptotic cells (Supplemental Fig. 2), the detected Vav1 was derived largely from macrophages.

The intracellular signaling elicited by efferocytosis is mediated by receptors or bridge proteins that recognize apoptotic cells. It was previously reported that Vav1 can be phosphorylated by the bridge protein Gas6, which cross-bridges apoptotic cells to macrophages through a receptor tyrosine kinase, Mer (51, 52). MFG-E8 is another protein that cross-bridges apoptotic cells to phagocytic macrophages. It can bind to PS on apoptotic cells and associate with αvβ3/5-integrin on the macrophages through an RGD motif. It was reported that Vav1 can be selectively phosphorylated at Y160 and activated after αvβ3-mediated adhesion on vitronectin and by the engagement of collagen, fibronectin, and fibrinogen (53, 54). Thus, we tested whether MFG-E8, a ligand for αvβ3, could also induce Vav1 phosphorylation. Indeed, peritoneal macrophages treated with mouse recombinant MFG-E8 (1 μg/ml) manifested a gradual increase in Vav1 phosphorylation, suggesting that Vav1 can be directly activated by MFG-E8 (Fig. 7C).

**Discussion**

The current study demonstrates that increased PtdIns(3,4,5)P3 signaling, caused by PTEN depletion, can enhance the ability of macrophages to clear apoptotic cells by elevating Rac GTPase activity. Using pharmacological inhibitors, we determined the roles of different PI3K isoforms in the process of efferocytosis. We found that the inhibition of p110α and p110β suppressed the engulfment of apoptotic cells, but the inhibition of p110γ did not cause any significant reduction in engulfment, indicating that signals from the GPCR signaling pathway that lead to p110γ-mediated PtdIns(3,4,5)P3 production are not involved in the engulfment of apoptotic cells (Fig. 1A). Because the Ser/Thr kinase Akt is an important downstream target of PtdIns(3,4,5)P3, we also investigated its role in efferocytosis. To our surprise, we found that neither pharmacological inhibition of Akt nor overexpression of
FIGURE 7. Increased Vav1 activation in PTEN−/− macrophages during efferocytosis. A, WT and PTEN−/− peritoneal macrophages were lysed, and Vav1 was immunoprecipitated. Pull-down eluates were analyzed by immunoblotting using anti-phospho-Tyr and anti-Vav1 Abs. B, Peritoneal macrophages were incubated with or without apoptotic cells at indicated time points. Unadhered cells were washed, and cell lysates were immunoprecipitated for Vav1 and analyzed by immunoblotting using anti-phospho-Tyr and anti-Vav1 Abs. Relative level of phosphorylated Vav1 to total Vav1 was quantified using ImageJ and depicted. C, Peritoneal macrophages were treated with 1 μg/ml MFG-E8 for indicated time points, and cell lysates were immunoprecipitated using Vav1 Ab. Levels of phosphorylated and total Vav1 were analyzed by immunoblotting using anti–phospho-Tyr and anti-Vav1 Abs as described above.

a constitutively activated form of Akt caused any change in the efficiency of efferocytosis, indicating that Akt may not play a key role in the engulfment of apoptotic cells.

The cellular levels of PtdIns(3,4,5)P3 can be regulated by lipid phosphatases; thus we reasoned that the dephosphorylation of PtdIns(3,4,5)P3 by PTEN may also regulate efferocytosis. To address this question, we studied the role of PTEN in efferocytosis using a myeloid-specific PTEN knockout mouse. We observed an increase in PTEN activity in macrophages during the engulfment of apoptotic cells. The mechanisms that regulate PTEN activation are not completely understood. The lipid phosphatase activity of PTEN could also be elevated directly by either MFG-E8 or Gas6, suggesting that the efferocytosis-associated PTEN activation can be elicited by multiple receptor signaling. PTEN activity can be regulated by various mechanisms. Phosphorylation of the 50-aa C-terminal tail domain has been proposed to be critical for protein stability and phosphorylation. PTEN can be phosphorylated by CK2 at three residues (S380, T382, and T383) in the C-terminal tail domain, which increases its activity but concomitantly reduces its stability (40, 41). It is possible that conformational changes induced by protein–protein interactions cause the phosphorylation of PTEN by CK2. It has also been shown that the inactivated form of the PI3K p110δ isoform could activate PTEN in a mechanism requiring RhoA activity (55, 56). It is possible that the downregulation of PI3K after its initial activation by the binding and engulfment of apoptotic cells may activate PTEN through a similar mechanism that requires RhoA and ROCK (57, 58). The exact nature of the association between Rho activation and PTEN activity during efferocytosis is a matter for further investigation.

The loss of PTEN in macrophages led to enhanced basal levels of PtdIns(3,4,5)P3, as inferred from the phosphorylation of Akt. PTEN−/− peritoneal macrophages engulfed both apoptotic neutrophils and thymocytes with higher efficiency than that of WT macrophages. Conversely, the forced expression of WT PTEN dramatically reduced the engulfment of apoptotic cells. These results imply that PTEN negatively regulates the engulfment of apoptotic cells. In vivo animal models also led to similar conclusions. In a dexamethasone-induced thymic involution model, the loss of PTEN resulted in the faster clearance of apoptotic thymocytes. In a peritonitis model involving the introduction of apoptotic cells into the peritoneum, PTEN−/− macrophages also engulfed apoptotic cells more efficiently (Fig. 4A).

The generation of apoptotic cells and efferocytosis are continuous processes. Clearance of apoptotic cells is a noninflammatory event. The roles of PtdIns(3,4,5)P3/Akt and PTEN signaling in the process of inflammation have been studied previously and remain controversial, with reports suggesting both pro- and inflammatory activities (44, 59). Recent studies have demonstrated that PtdIns(3,4,5)P3/Akt signaling exerts an anti-inflammatory effect in response to sepsis (60), bacterial pneumonia (33), and viral infections (61), which is likely mediated by the downregulation of NF-κB transcriptional activity through deactivation of GSK-3β upon Akt phosphorylation (42). Activation of PI3K–Akt also leads to a blockade of NF-κB activation in dendritic cells exposed to apoptotic cells (62). Consistent with these early reports, we also observed that the loss of PTEN led to increased GSK-3β phosphorylation, which may result in the modulation of NF-κB and increased production of anti-inflammatory cytokine IL-10. It appeared that PTEN disruption-induced elevation of PtdIns(3,4,5)P3 signaling was not sufficient to alter LPS-induced production of proinflammatory cytokines (IL-6 and TNF-α) in our experimental system. Nevertheless, PTEN disruption could both increase the production of IL-10 and reduce the production of IL-6 and TNF-α during efferocytosis. These results further imply that due to the enhanced anti-inflammatory response and the augmented efferocytic properties, PTEN−/− macrophages can clear apoptotic cells more efficiently under physiological conditions and may potentially lead to the faster resolution of inflammation. Thus, PTEN disruption can be a potential strategy for elevating efferocytosis in certain infectious and inflammatory diseases.

To analyze the molecular mechanisms driving enhanced efferocytosis, we focused on signaling events downstream of PtdIns(3,4,5)P3. Because efferocytosis was independent of Akt, we investigated the activation of other PH domain-containing proteins that can influence engulfment. PTEN has previously been shown to control FcγR-mediated phagocytosis by regulating Rac activation (63). We noted that the Vav1 exchange factor is activated upon efferocytosis and by MFG-E8 or Gas6. Apoptotic cells, via MFG-E8 or Gas6, activate class IA PI3K, leading to formation of PtdIns(3,4,5)P3 in macrophages. PtdIns(3,4,5)P3 in turn results in
Vav1 activation that peaks at 30 min after apoptotic cell stimulation. Vav1 activates Rac1 and F-actin polymerization leading to effector sites. Binding to apoptotic cells also leads to activation of PTEN that peaks and plateaus between 30 and 60 min to dephosphorylate PtdIns(3,4,5)P3, indicating that Vav1 and PTEN may function in the same pathway to regulate apoptotic cell engulfment. Conceivably, increased Vav1 activation in PTEN−/− macrophages enhances Rac activity and subsequent F-actin polymerization, leading to elevated engulfment of apoptotic cells.

In conclusion, our findings demonstrate that increased PtdIns(3,4,5)P3 signaling in PTEN-deficient mice results in enhanced clearance of apoptotic cells. This occurs through the regulation of Rac GTPase activation, which may be due to the augmented activity of Vav family guanine-nucleotide exchange factor, Vav1. In addition to increasing engulfment, depletion of PTEN may also lead to enhanced phosphorylation and inactivation of GSK-3β, which would lead to inactivation of NF-κB and subsequent suppression of the production of proinflammatory cytokines IL-6 and TNF-α and concurrent increase in expression of anti-inflammatory cytokine IL-10.

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apoptotic cell and Fc gamma receptor-mediated phagocytosis by macrophages.


