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PTP1B Deficiency Exacerbates Inflammation and Accelerates Leukocyte Trafficking In Vivo

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It is reported that PTP1B limits cytokine signaling in vitro. However, PTP1B’s function during inflammation in vivo is not known. In this report, we determined whether PTP1B deficiency affects allergic inflammation in vivo. Briefly, lungs of OVA-challenged PTP1B−/− mice had elevated numbers of eosinophils and eosinophil progenitors at 6 h after one OVA challenge and at 24 h after a third OVA challenge as compared with OVA-challenged wild-type mice. There was also an increase in numbers of CD11b+SiglecF+CD34+IL-5Rα+ eosinophil progenitors in the bone marrow, peripheral blood, and spleens of OVA-challenged PTP1B−/− mice. Intravital microscopy revealed that, in OVA-challenged PTP1B−/− mice, blood leukocytes rapidly bound to endothelium (5–30 min), whereas, in wild-type mice, blood leukocytes bound to endothelium at the expected 6–18 h. Consistent with early recruitment of leukocytes, lung eotaxin and Th2 cytokine levels were elevated early in the PTP1B−/− mice. Interestingly, spleen leukocytes from PTP1B−/− mice exhibited an increased chemotaxis, chemokinesis, and transendothelial migration in vitro. In summary, PTP1B functions as a critical negative regulator to limit allergic responses. The Journal of Immunology, 2012, 188: 874–884.
pathogen-free as determined in sentinel mice. Females of 6–8 wk of age were used in all experiments. The procedures were reviewed and approved by the Animal Care and Use Committee at the Northwestern University.

**Cells**

The endothelial cell line mHEVs, which was originally derived from male BALB/c mice, was cultured, as previously described (26, 27). Spleen cells were prepared from freshly isolated male PTP1B WT or PTP1B−/− spleens (26). Spleen RBCs were lysed by hypertonic shock (26).

**OVA-induced experimental atopic dermatitis model and intravital microscopy**

Mice were sensitized by i.p. injection of OVA 10 μg/μl aluminum hydroxide (alum; grade V; Sigma #A5503; Sigma-Aldrich) or saline/alum on days 0 and 7 (28). On day 17, mice were anesthetized by i.p. injection of averitin and challenged by intradermal injection of OVA (fraction VI; Sigma #A2512; Sigma-Aldrich) in saline in one ear or saline alone in the other ear. The mice were injected retro-orbitally with rhodamine 6G to label circulating leukocytes in the blood. The ears were shaved, challenged with 5 μg OVA in 10 μl PBS at the indicated time points (10 min, 30 min, 60 min, and 18 h), and then examined by intravital microscopy for leukocyte–endothelial cell interactions (numbers of leukocytes rolling, leukocyte rolling velocity, and leukocyte adhesion) as previously described (29). Intravital microscopy was performed using a 20× objective on an Olympus DSU microscope equipped with an OptiQuip Xenon light source (Olympus), a CCD camera, and Slidebook software (Intelligent Imaging Innovations). Adherent cells were scored as leukocytes that adhered to endothelium for >30 s and had a velocity of <5 μm/sec.

**OVA-induced allergic inflammation models**

We used a model of allergic inflammation to study leukocyte recruitment in PTP1B KO mice. The mice were sensitized by i.p. injection (200 μl of OVA grade V 10 μg/μl or saline/alum) on days 0 and 7 then challenged on days 15, 17, and 20 with intranasal OVA fraction VI (50 μg in 50 μl saline) or 50 μl saline alone (28). Where indicated, tissues were collected and examined either 15 min or 6 h after the first OVA challenge (day 16) or 24 h after the third OVA challenge (day 21). On day 16 or 21, the mice and their spleens were weighed. Spleen cells were isolated following standard procedures and further used for cytokopsin and flow cytometry analysis. The lungs were lavaged with 0.5 ml ice-cold PBS to obtain bronchoalveolar lavage (BAL) cells, and one lobe was harvested for cytokine/chemokine analysis; the rest of the lung was embedded in OCT and used for frozen tissue sections. BAL cells cytospins and blood eosinophils were incubated in a 7% CO2 incubator at 37˚C. After 2 h, plates were stained with fluorescent antibodies to identify the cell type and antigens of interest. Eosinophil peroxidase cytopsins were identified by characteristic morphology as previously described (30).

**Isolation of cells from the peripheral blood**

Peripheral blood cells were obtained by Ficoll-Hypaque density gradient centrifugation. Briefly, harvested blood was gently mixed with Ficoll-Paque Plus solution (density of <1.02 eu/ml; catalog number 1.07184; GE Healthcare) in a ratio 2:1, followed by centrifugation at 2000 rpm for 20 min at room temperature. The interphase containing WBCs was transferred to a new tube and washed twice in PBS by centrifugation at 1200 rpm for 10 min at 4˚C.

**Flow cytometry and immunofluorescence**

Peripheral blood cell FcRs were blocked by treating for 30 min at 4˚C with 5 μg/ml anti–CD16-CD32 (#553142; BD Pharmingen) in magnesium- and calcium-free Dulbecco’s PBS (Invitrogen) containing 2.5% (v/v) FCS, 1% BSA, and 0.05% sodium azide. Then, the cells were labeled using one of the following Ab combinations: 1) biotin-conjugated anti-CD11b (557395; BD Pharmingen) with a secondary label with PE-Cy7-conjugated streptavidin (#25-4317-82; eBioscience), PerCP-Cy5.5-conjugated anti–Gr1 (#552093; BD Pharmingen), PE-conjugated anti–Siglec-F (552126; BD Pharmingen), and FITC-conjugated anti-CD34 (#38-18917; Santa Cruz Biotechnology); 2) biotin-conjugated anti-CD11b (552095; BD Pharmingen) with a secondary label with PE-Cy7-conjugated streptavidin, PerCP-Cy5.5-conjugated anti–Gr1, PE-conjugated anti–Siglec-F, Alexa Fluor 488-conjugated anti-CD125 (IL-5Rα; #558553; BD Pharmingen), and Alexa Fluor 647-conjugated anti-CD193 (CCR3; #557074; BD Pharmingen); 3) allophycocyanin-conjugated anti–CD11b (#553512; BD Pharmingen), PerCP-Cy5.5-conjugated anti–Gr1, biotin-conjugated anti–Siglec-F (#552125; BD Pharmingen) with PE-Cy7-conjugated streptavidin, and PE-conjugated anti–I-AI-E (MHC class II [H2-A; #557000; BD Pharmingen]; and 4) PerCP-Cy5.5-conjugated anti–Lin (#556137; BD Pharmingen), PCy7-conjugated anti–Scal (BD Pharmingen #558162), PE-conjugated anti–Siglec-F (#552126; BD Pharmingen), Alexa Fluor 700-conjugated anti–CD34 (#560518; BD Pharmingen) and Alexa Fluor 488-conjugated anti–CD125 (IL-5Rα; #558553; BD Pharmingen). Live/dead violet fluorescent reactive dye kit (#L34595; Invitrogen) was used in all labeling combinations. Armenian hamster IgGl (#554007; BD Pharmingen) and rat IgG2a, κ (#558542; BD Pharmingen) were used as isotype-matched control Abs. Labeled cells were analyzed on a FACS caliber flow cytometer (BD Biosciences) with FACS Diva software (BD Biosciences). Flow cytometry data analysis was done with FlowJo software (Tree Star; Ashland, OR).

In addition, immunofluorescence labeling was performed on cytopsin of freshly isolated peripheral blood leukocytes following standard immunolabeling protocols. Abs used for labeling cytopsin were purified rat anti-mouse Siglec-F (#E50-2440; BD Pharmingen) with a secondary label with Alexa Fluor 658-conjugated goat anti-rat IgG (#A-11077; Molecular Probes) and Alexa Fluor 488-conjugated anti–CD125 (IL-5Rα; #558553; BD Pharmingen). Slides were mounted in ProLong Gold Antifade Reagent with DAPI (#P36935; Invitrogen) to visualize cell nuclear morphology. Confocal fluorescence and nonconfocal fluorescence micrographs were obtained with a 60× oil objective on a Zeiss LSM 510 META confocal microscope (Carl Zeiss).

**Cytokines, chemokines, and cell-surface markers**

The BAL supernatants and serum were examined for levels of cytokines IL-2, IL-4, IL-5, IL-10, IL-12, and IFN-γ using the Th1/Th2 Cyto kit Mouse 6-plex Panel of Invitrogen Luminex Protein Assays (#LMC0002; Invitrogen). The cytokines IL-4, IL-5, IL-13, IL-33, TSLP, and TNF-α, chemokines CCL11, CCL24, and MCP-1, adhesion molecules VCAM-1, P-selectin, and PECAM-1, and the leukocyte cell-surface markers Siglec-F, CD34, CD44, and IL-5Rα were determined by quantitative PCR from lung tissues. Leukocytes isolated by BAL and peripheral blood. Total RNA was isolated from 10–15 mg tissue or 1–5 million cells using the Qiagen RNeasy Mini Kit (Qiagen). cDNA was prepared from 500 ng mRNA/reaction using qScript cDNA Synthesis Kit (Quanta BioSciences) and analyzed by real-time PCR on a 7500 Real Time PCR System (Applied Biosystems) using TaqMan probes and TaqMan Universal Master Mix (Applied Biosystems). Amplification parameters were 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s alternating with 60˚C for 1 min. Expression of genes of interest was quantified as true gene copy numbers relative to copy numbers of the housekeeping gene (β-actin).

**In vitro cell adhesion and migration assays with laminar flow**

A parallel plate flow chamber was used to examine leukocyte migration across an endothelial cell line mHEVs under conditions of laminar flow of 2 dynes/cm², as previously described (27, 33). These endothelial cells are constitutively activated and express VCAM-1 and the chemokine MCP-1 that stimulate leukocyte transendothelial migration (26, 34). Moreover, this endothelial cell line functions similar to primary cultures of endothelial cells in that their signals through VCAM-1 have the same time course and magnitude of signals as primary endothelial cells (35). Leukocyte trans-endothelial migration was examined at 15 min. The number of spleen cells that were associated, but not migrated (phase light cells), at 15 min were scored. Thus, at that time point, the majority of nonmigrating cells roll off the monolayer of endothelial cells, as determined by microscopy (data not shown). Therefore, the number of spleen cells associated with the endothelial cells was quantified at 2 min of laminar flow, when these cells mediated cell–cell contact. The treatment groups for the adhesion and migration assays can only be compared within an experiment (each figure panel) because the adhesion and migration varies between experiments as we previously reported (26).

**In vitro chemotaxis and chemokinosis assays**

Spleen cells isolated from nonchallenged or OVA-challenged mice were used in transwell migration assays with cetuxin CCL11. Both lymphocytes and eosinophils express the CCL11 receptor CCR3 and are known to respond to CCL11. For chemotaxis, 1 × 10⁵ cells in 100 μl media were added to Transwell filter inserts (Corning COSTAR #3421, 5.0 μm pore size). CCL11 (200 pg/ml) in 37˚C 24-well plates that contained 600 μl media with 0.05 ng/ml recombinant murine CCL11 (#250-01; PeproTech). Thus, CCL11 was in the lower chamber. Then, transwells were incubated in a 7% CO2 incubator at 37˚C. After 2 h, plates were
challenged PTP1B

In contrast, in the OVA-stimulated WT mice (Fig. 2A), there was no recruitment of leukocytes in the lung lavage examined at 6 h after a single challenge with OVA. To examine the initial recruitment of leukocytes to the lung, mouse BAL was examined at 6 h after a single challenge with OVA. At this time point, there was no recruitment of leukocytes in the BAL of PTP1B WT and PTP1B−/− mice (Fig. 1A). However, eosinophils were significantly higher in the BAL (Fig. 1A), lung tissue (Fig. 1B–E), and blood (Fig. 1F) of OVA-challenged PTP1B−/− mice compared with WT mice. There was no difference in body weight among the groups (Fig. 1G). Therefore, deletion of PTP1B in vivo elevated OVA-induced lung inflammation.

Increased early recruitment of leukocytes in PTP1B−/− mice

To examine the initial recruitment of leukocytes to the lung, mouse lung lavage was examined at 6 h after a single challenge with OVA. At this time point, there was no recruitment of leukocytes in the OVA-stimulated WT mice (Fig. 2A). In contrast, in the OVA-challenged PTP1B−/− mice, there was a significant increase in all leukocyte cell types in the BAL as compared with the saline groups and the WT, OVA group (Fig. 2A). The largest number of infiltrating cells was eosinophil progenitor cells (Fig. 2A). In lung tissue, there was an increase in leukocytes in the OVA-challenged PTP1B−/− mice but not WT mice (Fig. 2B–G). The saline-treated PTP1B−/− did not have altered background levels of leukocytes (Fig. 2A, 2C). There was no effect on body weight (Fig. 2H).

In the blood, there was an OVA-dependent increase in total numbers of circulating mature blood eosinophils of OVA-challenged PTP1B−/− mice (Fig. 2D). In contrast, there was an OVA-independent increase in spleen size and total spleen cell counts of PTP1B−/− mice relative to the WT mice (Fig. 2J, 2K). Differential counts of cytopsins of PTP1B−/− spleen cells revealed significant increases in spleen numbers of lymphocytes, monocytes, eosinophil progenitor cells, and mature eosinophils (Fig. 2L). The increase in monocytes was OVA dependent, whereas the increase in lymphocytes and eosinophils was OVA independent in the spleen (Fig. 2L). In summary, the recruitment of leukocytes to the lungs of PTP1B−/− mice occurs rapidly, with recruitment as early as 6 h after the first OVA challenge; the lung and blood had Ag-dependent increases in inflammatory cells, whereas the spleen had both Ag-independent and Ag-dependent increases in leukocytes.

Increased production of inflammatory mediators in PTP1B−/− mice

We determined whether cytokines, chemokines, and adhesion molecules that regulate leukocyte infiltration in allergic inflammation were altered in PTP1B−/− mice. Tissue-derived cytokines TSLP and IL-33 were measured for their potential to orchestrate Th2-skewed early inflammatory responses. The cytokines IL-4 and IL-5, chemokines MCP-1, CCL11, and CCL24, and adhesion molecules P-selectin, VCAM-1, and PECAM-1 were examined because they stimulate neutrophil, monocyte, eosinophil, and lymphocyte infiltration. The cytokines TNF-α, IL-12, and IL-13 were measured as they contribute to allergic inflammation. Leukocyte cell-surface molecules α4-integrin (VLA4), IL-5Rα, SiglecF, CD44, and CD34 were measured as markers of leukocyte activation and migratory capacity. The cytokines IL-2, IL-10, and IFN-γ were examined because they downregulate signals for allergic inflammation.

At 6 h after a single OVA challenge, PTP1B−/− mice but not WT mice showed significant increases in lung expression of Th2 cytokines and chemokines compared with saline controls. Specifically, PTP1B−/− mice had significant OVA-dependent increases in the chemokines CCL11, CCL24, and MCP-1 and in cytokines IL-13, IL-33, IL-4, IL-5, IL-12, and IFN-γ in the lung tissue, lung lavage supernatant, or lung lavage cells, although the concentration of IFN-γ was extremely low relative to other cytokines (<2 pg/ml) (Fig. 3A, 3D, 3F). In contrast, there was no difference in OVA-induced TSLP, TNF-α, IL-2, and IL-10 in...
PTP1B−/− mice at 6 h after one OVA challenge (Fig. 3A, 3D, 3F). At 6 h after one OVA challenge, there was no change in lung tissue expression of the adhesion molecule VCAM-1 or P-selectin and a decrease in PECAM-1 in PTP1B−/− mice compared with WT mice (Fig. 3D). Interestingly, leukocytes isolated from peripheral blood of PTP1B−/− mice with one OVA challenge also exhibited significant increases in OVA-induced IL-5 and CCL11, mediators necessary for eosinophil recruitment and maturation in the lung (Fig. 3G). There were no changes in levels of α4-integrin expression (Fig. 3G). Expression of cell-surface receptors IL-5Rα, SiglecF, and CD34 and adhesion molecule CD44 were significantly increased on peripheral blood leukocytes of PTP1B−/− mice with one OVA challenge (Fig. 3G).

In contrast to our results obtained from one OVA challenge, there was a significant decrease after three OVA challenges in levels of cytokines IL-4, IL-5, and IL-10 in PTP1B−/− mice compared with WT mice (Fig. 3B). After the three OVA challenges, there was increased expression \( p < 0.05 \) of IL-13, IL-33, CCL-11, and CCL24 in the OVA-challenged WT mice compared with saline-treated WT mice (Fig. 3E). However, there was no difference in WT and PTP1B−/− mouse expression of the cytokines IL-2, IL-12, IL-13, and IL-33 and chemokines CCL11 and CCL24 (Fig. 3B, 3E). OVA-specific IgE Abs, which are produced during the OVA sensitization phase of allergic inflammation, were not altered in the PTP1B−/− mice (Fig. 3C). Therefore, there is a shift to early OVA-induced lung cytokine and chemokine production in the PTP1B−/− mice as compared with WT mice.

Intravital microscopy of leukocyte recruitment during allergic inflammation in PTP1B−/− mice

Leukocyte-endothelial cell interactions in vivo were quantified by intravital microscopy. Mice were sensitized with OVA i.p. on days 0 and 7. Then, 10 d later, the mice were challenged with OVA and examined by intravital microscopy. At the indicated time points (10 min, 30 min, 60 min, 6 h, 18 h), the mice were anesthetized, injected with rhodamine 6G i.v. to label leukocytes in the blood, challenged with OVA in the ear, and examined by intravital microscopy. For each mouse, one ear was challenged intradermally with OVA, whereas the other ear was challenged with saline (29). Adherent cells were scored as leukocytes, which adhered to endothelium for >30 s and had a velocity of <5 μm/sec. There was no difference between OVA-challenged or saline-treated WT and KO mice in numbers of leukocytes rolling or the velocity of the rolling leukocytes (Fig. 4A, 4B). Because rolling is mediated by
selectins and α4-integrin in its low-affinity state (36), the no difference in rolling is consistent with the no change in P-selectin or α4-integrin expression in Fig. 3D. However, firm adhesion of leukocytes to the endothelium peaked at 30 min in PTP1B−/− mice as opposed to 6 h in WT mice (Fig. 4C, 4D). This increased adhesion at 30 min by the PTP1B−/− mice is consistent with the increased expression of the chemokines MCP1, CCL11, and CCL24 (Fig. 3D–G). These chemokines are known to induce an increase in integrin affinity during leukocyte recruitment (37). There was no rolling or adhesion of leukocytes in the saline-treated ears of the OVA-sensitized WT or PTP1B−/− mice (data not shown).

Circulating leukocytes in PTP1B−/− mice express altered cell-surface markers

To compare leukocyte populations available for migration in PTP1B−/− and WT mice, peripheral blood leukocytes were examined for cell-surface markers that are related to migration and activation of leukocytes. CD11b+ myeloid cell populations were analyzed for expression of Siglec-F (murine eosinophil and hematopoietic progenitor markers), CD34 (progenitor marker), IL-5Rα (IL-5R and marker for eosinophil progenitors), CCR3 (CCL11 receptor), and MHC II (activation marker). Eosinophil precursors in the bone marrow were examined as Lin−Sca1−CD34+IL-5Rα+ eosinophil precursors at 6 h post-challenge, as measured by flow cytometry (Fig. 5D). CD11b+ SiglecFhigh/CD34+ cells in the blood were still elevated after three OVA challenges in the PTP1B−/− mice as compared with the OVA-challenged WT group (Fig. 5E). These CD11b+SiglecFhigh CD34+ cells in the blood cells expressed IL-5Rα (Fig. 5E). The majority of the eosinophils in the OVA-challenged WT mice are mature eosinophils with a CD11b+SiglecFhigh/CD34− phenotype (located below the R3 gates in Fig. 5B, 5C). Interestingly, the SiglecFhigh cells from PTP1B−/− mice also expressed MHC II (Fig. 5C). mRNA analysis of cells isolated from peripheral blood of PTP1B−/− mice at 6 h after one OVA challenge also showed increases in Siglec-F, CD34, IL-5Rα, and CD44 (Fig. 3G). Thus, there is an increase in numbers of circulating and migrating activated eosinophil progenitors in allergen-induced PTP1B−/− mice. Additionally, the bone marrow had increased numbers of Lin−Sca1−CD34+IL-5Rα+ eosinophil precursors at 6 h post-challenge, as measured by flow cytometry (Fig. 5D). CD11b+ SiglecFhigh/CD34+ cells in the blood were still elevated after three OVA challenges in the PTP1B−/− mice as compared with the OVA-challenged WT group (Fig. 5E).

In cytospins of peripheral blood cells (Fig. 6A) and spleen cells (Fig. 6B) from PTP1B−/− mice at 15 min after one OVA challenge, the eosinophils exhibited the characteristic ringed nuclei and lack of acidophilic granular staining of immature eosinophils (30, 31). This immature morphology was also predominant for eosinophils of BAL cells and spleens from PTP1B−/− mice at 6 h after one OVA challenge (Fig. 2A, 2L). Immunofluorescence la-

FIGURE 3. PTP1B deficiency increases lung cytokines and chemokines. A, At 6 h after one OVA challenge, BAL supernatants were examined for cytokine protein levels using the Th1/Th2 mouse cytokine multiplexing kit (Invitrogen). B, At 24 h after the last of three OVA challenges, BAL supernatant cytokines were examined using the Th1/Th2 mouse cytokine multiplexing kit. There was increased expression (p < 0.05) of IL-13, IL-33, CCL-11, and CCL24 in the OVA-challenged WT mice compared with saline-treated WT mice. C, At 24 h after the third OVA challenge, serum OVA-specific IgE was measured by ELISA. D, Six hours after one OVA challenge, lung tissue was preserved in RNAlater solution and then examined for cytokine and chemokine expression by real-time PCR. E, At 24 h after the third OVA challenge, BAL cells were preserved in RNAlater solution and then examined for cytokine and chemokine expression by real-time PCR. F, At 6 h after one OVA challenge, BAL cells were suspended in RNAlater and examined for cytokine and chemokine expression by real-time PCR. G, At 6 h after one OVA challenge, leukocytes isolated from peripheral blood were suspended in RNAlater solution and examined for cytokines, chemokines, and cell-surface molecules by real-time PCR. n = 6–8 mice per group. *p < 0.05 compared with WT, OVA group.
FIGURE 4. Intravital microscopy of leukocyte–endothelium interactions after OVA-challenge. Mice were sensitized with OVA/alum. At the indicated time points, mice received one OVA challenge in the ear. For this, mice were anesthetized and injected i.v. with rhodamine 6G to label blood leukocytes; the ears were shaved, challenged with OVA, and examined by intravital microscopy. A, Number of rolling cells per blood vessel. B, Velocity of rolling leukocytes in blood vessel lumens. C, Number of leukocytes firmly adhered to blood vessel walls. D, Single-frame images (original magnification ×20) taken during intravital microscopy showing leukocyte–endothelium interactions in ear blood vessels of OVA-challenged mice. Adherent leukocytes (white arrows) peak at 30 min in OVA-challenged PTP1B-deficient mice as opposed to 6 h in OVA-challenged WT mice. Saline-challenged ears of OVA-sensitized mice did not show recruitment of leukocytes (data not shown). n = 2 vessels from 3 to 4 mice per time point. *p < 0.05 compared with WT control.

In summary, there is an increased pool of eosinophil progenitors in the bone marrow, spleens, and blood of PTP1B−/− mice, which is consistent with the early accumulation of the eosinophil progenitors in the lungs of OVA-challenged PTP−/− mice.

Spleen leukocytes from PTP1B−/− mice have an increased capacity for transendothelial migration in vitro

To determine whether the PTP1B−/− leukocytes have an enhanced capacity for transendothelial migration, we performed in vitro adhesion and migration assays with WT endothelial cells under physiological laminar flow. In this assay, the leukocyte adhesion and transendothelial migration are dependent on VCAM-1 and chemokines produced by the endothelial cells (26, 34). The treatments in these assays can only be compared within an experiment (each figure panel) because the adhesion and migration vary between experiments as we previously reported (26). Spleen leukocytes from nonchallenged PTP1B−/− mice had increased VCAM-1-dependent adhesion to and migration across WT endothelial cells (Fig. 7A, 7C). Remarkably, after just 2 min of laminar flow in the adhesion assay, some PTP1B−/− leukocytes had migrated through the endothelial cell monolayers (data not shown). Spleen leukocytes collected from PTP1B−/− mice at 15 min after one OVA challenge migrated in significantly greater numbers than spleen cells from WT mice (Fig. 7D), but they did not have altered initial adhesion (Fig. 7B). We also examined spleen leukocyte chemotaxis and chemokinesis in response to CCL11. Spleen leukocytes collected from PTP1B−/− mice at 15 min after OVA challenge exhibited significantly greater CCL11-driven chemotaxis and chemokinesis compared with OVA-challenged WT controls (Fig. 7E, 7F). In summary, leukocytes isolated from spleens of PTP1B−/− mice exhibited a greater capacity for chemotaxis, chemokinesis, and transendothelial migration in vitro.

Discussion

In this study, PTP1B deficiency in mice exacerbated allergen-induced inflammation through early recruitment of leukocytes and, in particular, eosinophil progenitors. This was accompanied by uncontrolled production of proinflammatory mediators, which resulted in increased eosinophilia in the lung. However, there was no difference in the number of resident leukocytes in the lungs of nonchallenged PTP1B−/− mice and WT mice. This report has important implications for PTP1B in the negative regulation of allergic responses.

In the PTP1B−/− mice, allergen challenge induced rapid recruitment of leukocytes. It has been reported that the kinetics for recruitment of leukocytes in mice vary based on the site, dose, and frequency of allergic sensitization and allergen challenge. There is a sequential accumulation of leukocyte cell types in the...
lung during the response to OVA challenge, consisting of an early accumulation of infiltrating macrophages or neutrophils (within hours) and a later increase of T lymphocytes and eosinophils (within days). Eosinophils, a hallmark of allergic inflammation, increase at 3 h after each allergen challenge, but the peak in eosinophils occurs at 3 to 4 d after several Ag challenges (41–44). The recruitment of eosinophils is regulated by Th2 cytokines and eotaxin in the BAL fluid and lung tissue. During the resolution of allergic inflammation, there is a decline of eosinophil numbers in BAL fluid starting at 7 d after challenge and a complete decline by 14 d (42–44). At 6 h following only one allergen challenge or at 24 h after the third allergen challenge, there was a significant increase in eosinophils in OVA-challenged PTP1B−/− compared with WT allergen-challenged mice. Allergen-challenged PTP1B−/− mice also exhibited splenomegaly and increased total blood eosinophil counts, indicative of underlying elevated hematopoiesis. Eosinophil progenitors, which have the characteristic ringed nuclei and lack acidophilic granular staining of mature eosinophils (30, 31), were the most frequent cells in BAL infiltrate, followed by neutrophils and macrophages, in the OVA-challenged PTP1B−/− mice. In addition, there were elevated numbers of eosinophil progenitors available for recruitment into the tissue because immature eosinophils were also in significantly greater quantities in the bone marrow, spleens, and peripheral blood of allergen-challenged PTP1B-deficient mice as compared with controls. Mature eosinophils and lymphocytes were detected in the PTP1B-deficient mice, although their numbers were low.

These findings are consistent with the concept that upon allergen challenge, CD34+ hematopoietic eosinophil progenitor cells are released from the bone marrow to peripheral circulation and migrate to the sites of inflammation. It is the combination of CD34+ and IL-5Rα+ expression that is characteristic for defining eosinophil progenitors (45), with IL-5Rα being highly expressed on immature but not mature eosinophils (46, 47). CD34 expression is also high on immature eosinophils but low on mature murine eosinophils (39). The CD34+IL-5Rα+ eosinophil progenitors are reported to be transiently elevated in the bone marrow and lung at 6 h after inhaled allergen challenge, returning to preallergen levels at 12 h (44). It is also reported that Siglec-F is expressed by eosinophils (48, 49) and bone marrow immature myelomonocytic cells (50, 51). We have made the novel observation by flow cytometry that high expression of Siglec-F correlated with high expression of CD34 in PTP1B−/− mice. In the PTP1B−/− mice, OVA challenge induced a rapid increase in Siglec-FhighCD34+IL-5Rα+ leukocytes in the bone marrow, spleens, and peripheral blood, whereas the numbers of these Siglec-FhighCD34+IL-5Rα+ eosinophils remained low in WT mice. The eosinophils in the OVA-challenged WT mice were primarily mature eosinophils (SSChighCD11b–SiglecFlowCD34−) after three OVA challenges. Consistent with the flow cytometry data, immunofluorescence labeling of cytosins prepared from spleens and peripheral blood of PTP1B−/− mice as soon as 15 min after challenge also demonstrated an increase in Siglec-FhighCD34+IL-5Rα+ eosinophil progenitors with low cytoplasmic granularity and characteristic eosinophil nuclear morphology. Siglec-FlowIL-5Rα+ eosinophils with well-defined cytoplasmic granularity (likely mature eosinophils) were also in increased numbers in the cytosins of spleen cells from PTP1B−/− mice as compared with WT mice. Leukocytes isolated from peripheral blood and BAL fluid of PTP1B−/− mice showed an increase in mRNA for CD34 and IL-5Rα. Thus, there was an increase in numbers of circulating and migrating progenitor eosinophils as well as an increase in mature eosinophils in OVA-challenged PTP1B−/− mice.

Once at the site of inflammation, it has been suggested that progenitor cells can participate in the development of inflammation by maturing, locally, into inflammatory effector cells in response to the factors released in situ (47, 52, 53). In our study with the PTP1B−/− mice, the overexpression of IL-33, a tissue-derived cytokine, may contribute to this process, because it has been re-
ported that IL-33 directly stimulates eosinophil differentiation from progenitors in an IL-5–dependent manner and that IL-33 stimulates eosinophil progenitor production of IL-5 and IL-13 (52, 54, 55). Differentiation and function of eosinophils and their progenitors is also influenced by eotaxins produced at the site of allergic inflammation, because eotaxins have been shown to increase eosinophil survival and differentiate eosinophil progenitors into mature eosinophils independently of IL-5 (53). We showed by quantitative PCR analysis that BAL cells had increased production of IL-4, IL-5, and MCP-1 at 6 h after allergen challenge, suggesting early effector functions of newly migrated cells. Interestingly, PTP1B−/− leukocytes isolated from peripheral blood of OV A-challenged mice also had elevated levels of IL-5 and CCL11 (eotaxin 1). Therefore, the increase in mature eosinophils in the lungs of PTP1B−/− mice with three OV A challenges may at least in part be a direct result of in situ maturation of eosinophil progenitors that were recruited to the lung in early stages of inflammation.

The intravital microscopy studies with the allergen-challenged PTP1B−/− mice demonstrate adherence of leukocytes within minutes, which is in contrast to the 6 h delay for leukocyte adherence in the allergen-challenged WT mice. Such a rapid capacity for recruitment of PTP1B−/− leukocytes has not been reported. Moreover, in vitro migration assays under laminar flow conditions, there was increased transendothelial migration of leukocytes isolated from spleens of PTP1B−/− mice through monolayers of WT endothelial cells producing MCP-1. There was also increased CCL11-stimulated chemotaxis and chemokinesis by the leukocytes from allergen-challenged PTP1B−/− mice. Thus, the PTP1B−/− leukocytes have an increased capacity for migration. This increased adhesion by intravital microscopy is consistent with the rapid increase in expression of chemokines in the PTP1B−/− mice, because chemokines activate chemokine receptor signaling for the phosphorylation and increased affinity of integrins (56), and it is known that the high-affinity state of integrins mediates firm adhesion (37).

Further explanation for the increased migration properties of PTP1B-deficient cells may be their priming for migration as a result of elevated expression of IL-5 and CCL11 in the bone marrow or peripheral blood. It is reported that an increase in eosinophil progenitor numbers in tissues immediately following allergen stimulation can be the consequence of local proliferation of CD34+ cells in response to IL-5 (47). Interestingly, studies using CD34-deficient bone marrow transplants demonstrate a dramatic reduction in Ag-induced eosinophil and mast cell infiltration into airways, suggesting that hematopoietic CD34 expression is a pre-

**FIGURE 6.** Cytospins and immunolabeling of peripheral blood leukocytes. A, Representative micrographs of DiffQuick-stained cytospins of peripheral blood leukocytes from PTP1B−/− mice and WT mice at 15 min after one OVA challenge. White arrows, mature eosinophils; black arrow, eosinophil progenitors. B, Cytospins of leukocytes from spleens of PTP1B−/− mice and WT mice at 15 min after one OVA challenge were labeled with DAPI to identify nuclear morphology. Cells were also immunofluorescence-labeled with anti–Siglec-F (Alexa Fluor 568) and anti–IL-5Rα (FITC). Representative fluorescent and phase-contrast micrographs are shown. The top two rows of panels are fluorescent images of spleen cells from WT, OVA and PTP1B−/−, OVA mice, respectively (original magnification ×60). The bottom row of panels are images from a confocal optical slice through the center of the cells; these are zoomed confocal images collected from the same cells as those indicated in the dashed boxes in the center panel of images; this demonstrates cytoplasmic expression of IL-5Rα and Siglec-F in eosinophil progenitors. Red arrows, representative mature eosinophil; yellow arrows, representative eosinophil progenitor. C and D, The mature eosinophils (red arrows in B) and eosinophil progenitors (yellow arrows in B) were counted from immunofluorescence-labeled cytospin preparations as in B. n = 6–8 animals/group. C, Percent of spleen cells. *p < 0.05 compared with the WT groups. D, Total number of cells in mouse spleens. *p < 0.05 compared with the WT, saline group. **p < 0.05 compared with the other groups.
Remarking, PTP1B with spleen cells isolated from mice at 15 min after one OV A challenge. with 50 ng eotaxin-1/ml for 2 h. F motaxis of spleen cells isolated from nonchallenged mice and from mice at 15 min after one OV A challenge. Leukocyte migration was examined at 15 min of laminar flow. A, Adhesion assay with spleen cells isolated from nonchallenged mice. Spleen cells associated with the endothelial cells were counted if they mediated cell–cell contact at 2 min of laminar flow. B, Adhesion assay with spleen cells isolated from mice at 15 min after one OVA challenge. Remarkably, PTP1B−/− cells not only mediated cell–cell contact in the 2 min of laminar flow, but many of them also migrated through WT endothelial cell monolayers within the 2 min of laminar flow. C, Migration assay with spleen cells isolated from nonchallenged mice. Leukocyte migration was examined at 15 min of laminar flow. D, Migration assay with spleen cells isolated from mice at 15 min after one OVA challenge. Leukocyte migration was examined at 15 min of laminar flow. E, Chemotaxis of spleen cells isolated from nonchallenged mice and from mice at 15 min after one OVA challenge. Chemotaxis assay was performed with 50 ng eotaxin-1/ml for 2 h. F, Chemokinesis of spleen cells isolated from nonchallenged mice and at 15 min after one OVA challenge. The chemokinesis assay was performed with 50 ng eotaxin/ml for 2 h. n = 3 animals per group. *p < 0.05 compared with WT; OVA control.

FIGURE 7. Leukocytes isolated from spleens of PTP1B KO mice have increased capacity for migration, chemotaxis, and chemokinesis in vitro. A. Adhesion assay with spleen cells isolated from nonchallenged mice. B. Adhesion assay with spleen cells isolated from mice at 15 min after one OVA challenge. C. Migration assay with spleen cells isolated from nonchallenged mice. D. Migration assay with spleen cells isolated from mice at 15 min after one OVA challenge. E. Chemotaxis of spleen cells isolated from nonchallenged mice and from mice at 15 min after one OVA challenge. F. Chemokinesis of spleen cells isolated from nonchallenged mice and at 15 min after one OVA challenge.

required for development of allergic asthma (39). Moreover, expression of CD34 has been shown to enhance cell mobility and invasiveness (39, 57). CD34+ progenitor cells are recruited to the sites of inflammation in allergic diseases through many of the same adhesion receptors used for progenitor cell homing to the bone marrow, such as PSGL-1, α4β1 integrin, and CD44 (58). We detected increased expression of CD34 and CD44, but no change in α4β1 integrin expression on PTP1B−/− leukocytes isolated from peripheral blood cells. Consistent with a role for CD44 in recruitment, CD44-deficient mice in models of chronic inflammatory disease have significantly reduced leukocyte recruitment (59). Additionally, the Siglec-Fhigh/IL5Rxα+ progenitors in the PTP1B−/− mice are in a high activation state because they have increased expression of MHC II. These findings are in agreement with reports that eosinophil progenitors also serve as rapidly mobilized APCs, which further contribute to development of inflammation (60, 61). Based on our findings, we suggest that in addition to in situ proliferation, cytokine-primed Siglec-Fhigh CD34+IL5Rxα+ cells are equipped with cell-surface markers that enable their quick mobilization in peripheral blood circulation and very rapid recruitment to tissues in OVA-challenged PTP1B−/− mice.

Involvement of PTP1B in limiting cytokine regulatory pathways is consistent with the increased recruitment of eosinophils and their progenitors and resulting hypereosinophilia in the lungs of OVA-challenged PTP1B−/− mice. Specifically, it is reported that PTP1B constitutively suppresses STAT activity through tyrosine dephosphorylation, thus participating in negative regulation of JAK/STAT signaling pathways (62). Signaling through JAK/STAT6 occurs during allergic inflammation. Eosinophil recruitment and Th2 cytokine production are regulated by Stat6-dependent genes in hematopoietic non-T cells (63). Stat6 also regulates the differentiation of monocytes to alternatively activated macrophages, and the absence of these macrophages prevents eosinophil recruitment to lung (61). In addition, eotaxin expression has been shown to be regulated by the Th2 cytokines IL-4 and IL-13 via a STAT6-dependent pathway (64). Thus, loss of PTP1B function elevates cytokine signaling and cytokine production.

Elevated eotaxin, IL-5, and eosinophil recruitment in the PTP1B−/− mice is consistent with studies in which overexpression of IL-5 or eotaxins elevates eosinophil recruitment (65, 66). IL-5 functions in recruitment of progenitors; peak levels of IL-5 correspond to CD34+IL5Rxα+ progenitor levels at 6 h after allergen challenge (44). This IL-5–mediated enhancement of leukocyte recruitment is consistent with the elevated IL-5 and eosinophil recruitment that we observed in PTP1B−/− mice. IL-5 also increases expression of IL-5Rxα and CCR3 on the surface of CD34+ cells (67). Unlike mature eosinophils, progenitors maintain prolonged expression of IL-5Rxα, indicating that immature cells are better equipped to respond to ongoing exposure to IL-5. Although IL-5 defines progenitor’s commitment to eosinophil lineage and enables mobilization from the bone marrow, eotaxin (CCL11) stimulates progenitor cell’s migration responses (68). Eotaxin is known to induce a rapid chemotaxis of eosinophils and their progenitors from the bone marrow in the presence of IL-5 (65, 69). IL-5 induces the expression of the eotaxin receptor CCR3 on eosinophils. Consistent with elevated IL-5 in the PTP1B−/− mice, we found increased expression of CCR3 on CD34+IL5Rxα+ cells in OVA-challenged PTP1B−/− mice. Higher numbers of CCR3 receptors on PTP1B−/− progenitor cells would contribute to the greater migration of these cells in response to the eotaxins, CCL11 and CCL24. The early increase in expression of cytokines, cytokine receptors, and chemokines in the OVA-challenged PTP1B−/− mice is consistent with enhanced leukocyte recruitment in these mice.

At 24 h after the third OVA challenge in PTP1B-deficient mice, there was a significant decrease in both message and protein levels of IL-4, IL-5, and IL-10 and no difference in IL-2, IL-12, IL-13, IL-33, and eotaxin levels in OVA-challenged compared with WT mice. Reports of cytokine kinetics show peak of IL-4 and IL-5 BAL protein levels at 6–12 h, which return to preallergen levels by 48 h after the last challenge. It is also reported that after allergen challenge, eotaxin is elevated from 2 to 48 h, peaking at 24 h and
and the recruitment of leukocytes during allergic responses. Functions to limit induction of allergic inflammatory mediators in nonchallenged lungs of PTP1B-deficient mice. Thus, PTP1B activation, chemokinesis, and chemotaxis and are capable of endothelial cell responses in murine models of asthma. Am. J. Physiol. Lung Cell. Mol. Physiol. 229: L1111–L1125.


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Corrections


The fifth author’s name was omitted from the article. The corrected author and affiliation lines are shown below.

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