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PTP1B Deficiency Exacerbates Inflammation and Accelerates Leukocyte Trafficking In Vivo

Sergejs Berdnikovs,* Vladimir I. Pavlov,† Hiam Abdala-Valencia,* Christine A. McCary,* David J. Klumpp,‡ Michel L. Tremblay,‡ and Joan M. Cook-Mills*

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.

Protein tyrosine phosphatase 1B (PTP1B), which is ubiquitously expressed, regulates cellular signaling (1). PTP1B dephosphorylates the insulin receptor, and, therefore, PTP1B−/− mice exhibit insulin hypersensitivity (2, 3), and patients with elevated PTP1B activity exhibit insulin insensitivity (4–8). PTP1B also regulates leptin receptor signaling by dephosphorylating the receptor-associated kinase Jak2 to limit the generation of high-fat diet-induced obesity (2). Because PTP1B is considered to be a target for the treatment of type 2 diabetes and obesity, there are multiple high-affinity PTP1B inhibitors in development (9, 10). A component of these metabolic disorders is inflammation (11–13). Moreover, obesity frequently coincides with allergic inflammatory diseases such as asthma (14–17). Although it is known that PTP1B expression is induced by inflammation in vivo (18), and it is suggested that PTP1B may be a target of anti-inflammatory therapies, the effect of PTP1B inhibition on inflammation in vivo in allergic disease is not known.

Reports indicate that, in vitro, PTP1B is involved in control of immune cell signaling. PTP1B is a negative regulator of cytokine receptors and receptor tyrosine kinases in lymphohematopoietic cells. PTP1B controls cytokine signaling pathways by its negative action on the JAK/STAT pathway, dephosphorylating JAK2, TYK2, and STAT5a/b (3, 19). Phosphorylated STAT6 may also serve as a cytoplasmic substrate for PTP1B because overexpression of PTP1B leads to STAT6 dephosphorylation and the suppression of STAT6 transcriptional activity, whereas PTP1B deficiency increases IL-4–induced STAT6 signaling in B cells (20). Knockdown of endogenous PTP1B expression increases production of TNF-α, IL-6, and IFN-β in TLR-triggered macrophages (21). Macrophages isolated from PTP1B−/− animals are highly sensitive to IFN-γ, as demonstrated by increased phosphorylation of Stat1 (22). PTP1B is also known to regulate myeloid cell proliferation and differentiation; there are increased numbers of committed macrophage precursors, as well as splenic monocytes and granulocytes, in PTP1B−/− mice, attributable to reduced apoptosis of these cells and increased numbers of splenic CFU-GM (23, 24). Selective expansion of monocytes is observed in the spleens of older PTP1B−/− mice (24). PTP1B−/− cells display increased inflammatory activity in vitro and in vivo through the constitutive upregulation of activation markers as well as increased sensitivity to endotoxin (24). Other studies indicate that PTP1B deficiency increases migratory capacity of PTP1B-deficient fibroblasts in vitro (25). Collectively, these previous reports point to participation of PTP1B in the control of inflammatory processes, although no studies have addressed leukocyte recruitment for the development of inflammation in PTP1B−/− mice.

In the current study, we demonstrate for the first time, to our knowledge, that PTP1B deficiency exacerbates allergic inflammation and leukocyte recruitment in vivo during OVA-induced allergic inflammation and atopic dermatitis. The mechanisms responsible for increased allergic inflammation in these mice include elevated hematopoietic cell production, early recruitment of leukocytes to the inflammatory site, upregulation of leukocyte chemokine and cytokine receptors, and highly elevated production of chemokines and Th2 cytokines. These data suggest that PTP1B functions as a critical negative regulator of inflammatory responses.

Materials and Methods

Animals

PTP1B wild-type (WT) and knockout (KO) mice (BALB/c background) were from Dr. Michel Tremblay (McGill University, Montreal, QC, Canada). The mice were housed under barrier conditions and were specific

Abbreviations used in this article: alum, aluminum hydroxide; BAL, bronchoalveolar lavage; KO, knockout; MHC II, MHC class II; PTP1B, protein tyrosine phosphatase 1B; WT, wild-type.

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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 mHEVas, 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<sup>+/−</sup> spleens (26). Spleen RBCs were lysed by hypotonic shock (26).

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

Mice were sensitized by i.p. injection of OVA 10 μg/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 averitn 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 Optiphot 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/alum or saline/alum on days 0 and 7, and 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 cytokins 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 DiffQuick (Dade Behring) stained and counted according to standard morphological criteria. Eosinophil precursors in the lung cytospins were identified by characteristic morphology as previously described (30, 31). OVA-specific IgE was determined by ELISA, as previously described (32). Airway morphology and leukocyte infiltrate were examined by H&E staining following standard staining protocols for frozen lung tissue sections.

**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 1.077 g/ml; catalog number se-75184; GE Healthcare) in a ratio 2:1, followed by centrifugation at 2000 rpm for 20 min at room temperature. The interface 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 0.01 M 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, PE-conjugated anti–I-A/I-E (H-2M class II [MHC II]; #557000; BD Pharmingen); and 4) PE-conjugated anti–I-A/I-E (H-2M class II; #557000; BD Pharmingen) and rat IgG<sub>2a</sub> κ (#558542; BD Pharmingen) were used as isotype-matched control Abs. Labeled cells were analyzed on a FACSanu flow cytometer (BD Biosciences) with FACSDiva software (BD Biosciences). Flow cytometric data analysis was done with FlowJo software (Tree Star; Ashland, OR).

In addition, immunofluorescence labeling was performed on cytospins of freshly isolated peripheral blood leukocytes following standard immuno- labeling protocols. Abs used for labeling cytospins were purified rat anti-mouse Siglec-F (#554007; BD Pharmingen) and rat IgG<sub>2a</sub> κ (#558542; BD Pharmingen) were used as isotype-matched control Abs. Labeled cells were analyzed on a FACSanu flow cytometer (BD Biosciences) with FACS Diva software (BD Biosciences). Flow cytometric data analysis was done with FlowJo software (Tree Star; Ashland, OR).

**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 Cytokine Mouse 6-plex Panel of Invitrogen Luminex Protein Assays (#LMC0002; Invitrogen). The cytokines IL-4, IL-5, IL-13, 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 leukocytes isolated from BAL and peripheral blood. Total RNA was isolated from 10–15 mg lung tissue or 1–5 million cells using the Qiagen RNAeasy 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 mHEVas under conditions of laminar flow of 2 dynes/cm<sup>2</sup>, 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 counted because, 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 cytokin CCL11. Both lymphocytes and eosinophils express the CCL11 receptor CCR3 and are known to respond to CCL11. For chemotaxis, 1 × 10<sup>5</sup> cells in 100 μl media were added to Transwell filter inserts (Corning COSTAR #3421, 5.0 μm pore size; Corning) and placed 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% CO<sub>2</sub> incubator at 37°C. After 2 h, plates were
tapped gently to dislodge any loosely attached cells, and cells collected from the lower chamber (the plate well) were counted using a hemocytometer. For chemokinesis, 0.05 ng/μl recombinant murine CCL11 was added to the upper well of Transwell filter inserts containing the cells. Cells were collected at 2 h and counted, and n = 6 animals/group.

**Statistical analysis**

Data were analyzed by a one-way ANOVA, followed by Tukey’s multiple comparisons test (SYSTAT 11). All data are presented as the means ± SEM.

**Results**

*Increased eosinophilia in PTP1B<sup>−/−</sup> mice during allergic lung inflammation*

We examined whether PTP1B KO mice had altered leukocyte recruitment during allergic inflammation. For this model, mice were sensitized with OVA/alum on days 0 and 7 and then challenged with OVA in saline on days 15, 17, and 20. Twenty-four hours after the last OVA challenge, leukocytes were examined in the blood, BAL, and lung tissue. OVA induced the recruitment of eosinophils in the BAL of PTP1B WT and PTP1B<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> mice but not WT mice (Fig. 2B–G). The saline-treated PTP1B<sup>−/−</sup> 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<sup>−/−</sup> mice (Fig. 2D). In contrast, there was an OVA-independent increase in spleen size and total spleen cell counts of PTP1B<sup>−/−</sup> mice relative to the WT mice (Fig. 2J, 2K). Differential counts of cytopsins of PTP1B<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> mice*

We determined whether cytokines, chemokines, and adhesion molecules that regulate leukocyte infiltration in allergic inflammation were altered in PTP1B<sup>−/−</sup> 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<sup>−/−</sup> mice but not WT mice showed significant increases in lung expression of Th2 cytokines and chemokines compared with saline controls. Specifically, PTP1B<sup>−/−</sup> 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 a significant decrease (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
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 (Qiagen) and examined for cytokine, chemokine, and adhesion molecule 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.

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), C34 (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+ progenitors (38). In the peripheral blood of the OVA-challenged PTP1B−/− mice, there was an additional distinct population of CD11b"Siglec-Fhigh" cells as compared with WT mice (Fig. 5A). This population of cells also expressed CD34 and expressed IL-5Rα and CCR3, indicating that these cells are progenitors with eosinophil commitment (Fig. 5B, 5C). Interestingly, the Siglec-Fhigh 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, C34, 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). 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, 5E). This is consistent with reports that mature eosinophils have very low CD34 expression (39). The percentage of CD11b"SiglecFhigh"CD34"IL-5Rα+ eosinophil progenitors in peripheral blood of PTP1B−/− mice after three OVA challenges was significantly higher than in the WT controls (Fig. 5F).

In cytopsins 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-
mice per time point. *p < 0.05 compared with WT mice.

OV A-sensitized mice did not show recruitment of leukocytes in ear blood vessels of OV A-challenged mice as opposed to 6 h in WT counterparts (Fig. 6D). 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 allergen sensitization and allergen challenge. There is a sequential accumulation of leukocyte cell types in the bone marrow, spleen, and peripheral blood cytospins also identified these cells as eosinophil progenitors; in cytospins, there were Siglec-Fhigh IL-5Rx− eosinophil progenitors with low cytoplasmic granularity and heterochromatin condensation (Fig. 6B, yellow arrow). There were also Siglec-Fhigh IL-5Rx− mature eosinophils with well-defined cytoplasmic granularity (Fig. 6B, red arrow). The SiglecF staining is intracellular as shown in the confocal immunofluorescence optical xy-slices through the middle of the eosinophils (Fig. 6B); this is consistent with reports that SiglecF has intracellular functions (40). The percentage of eosinophil progenitors in the spleen was increased in the PTP1B−/− OV A-challenged and in the nonchallenged mice compared with their WT counterparts (Fig. 6C). Furthermore, the total number of progenitor and mature eosinophils was increased in spleens of PTP1B−/− OV A-challenged mice as compared with the WT, OV A-challenged WT controls (Fig. 6D). There was also an increase in total number of eosinophil progenitors in the PTP1B−/− spleen leukocytes collected from PTP1B−/− mice at 15 min after one OV A 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 OV A challenge exhibited significantly greater CCL11-driven chemotaxis and chemokinesis compared with OV A-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.

In intravital microscopy of leukocyte–endothelium interactions after OV A-challenge. Mice were sensitized with OV A/alum. At the indicated time points, mice received one OV A 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 OV A, 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 OV A-challenged mice. Adherent leukocytes (white arrows) peak at 30 min in OV A-challenged PTP1B−/− deficient mice as opposed to 6 h in OV A-challenged WT mice. Saline-challenged ears of OV A-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.

**FIGURE 4.** Intravital microscopy of leukocyte–endothelium interactions after OV A-challenge.
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 mature myelomonocytic cells (50, 51). We have made the novel observation by flow cytometry that high expression of Siglec-F is correlated with high CD34 expression in PTP1B−/− mice. In the PTP1B−/− mice, OVA challenge induced a rapid increase in Siglec-F+CD34+IL-5Rα- leukocytes in the bone marrow, spleens, and peripheral blood, whereas the numbers of these Siglec-F+CD34-IL-5Rα- eosinophils remained low in WT mice. The eosinophils in the OVA-challenged WT mice were primarily mature eosinophils (SSC<sub>high</sub>CD11b<sup>+</sup>SiglecF<sup>−/−</sup>CD34<sup>−/−</sup>IL-5Rα<sup>−/−</sup>) three OVA challenges. Consistent with the flow cytometry data, immunofluorescence labeling of cytoinks prepared from spleens and peripheral blood of PTP1B−/− mice as soon as 15 min after challenge also demonstrated an increase in Siglec-F<sup>high</sup>CD34<sup>−/−</sup>IL-5Rα<sup>−/−</sup> eosinophil progenitors with low cytoplasmic granularity and characteristic eosinophil nuclear morphology. Siglec-F<sup>low</sup>IL-5Rα<sup>−</sup> eosinophils with well-defined cytoplasmic granularity (likely mature eosinophils) were also in increased numbers in the cytoinks 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 OVA-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 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 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-
Remarkably, PTP1B with spleen cells isolated from mice at 15 min after one OVA challenge. From peripheral blood cells. Consistent with a role for CD44 in with 50 ng eotaxin-1/ml for 2 h. The chemotaxis assay was performed with spleen cells isolated from nonchallenged mice and from mice at 15 min after one OVA challenge. Leukocyte migration was examined at 15 min of laminar flow. Adhesion assay with spleen cells isolated from mice at 15 min after one OVA challenge. The chemokinesis assay was performed with 50 ng eotaxin/ml for 2 h. *p < 0.05 compared with WT, OVA control.

At 24 h after the third OVA challenge in PTP1B−/− 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

Involvement of PTP1B in limiting cytokine regulatory pathways is consistent with the increased recruitment of eosinophils and their progenitors and resulting hyper eosinophilia 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 over-expression 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+IL-5Rα+ 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-5Rα and CCR3 on the surface of CD34+ cells (67). Unlike mature eosinophils, progenitors maintain prolonged expression of IL-5Rα, 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+IL-5Rα+ 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.

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. 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. The chemotaxis assay was performed with 50 ng eotaxin-1/ml for 2 h. F, Chemokinesis of spleen cells isolated from nonchallenged mice and mice 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.
returning to control levels by 72 h (42, 44). Thus, increased eo-
sinophilia with decreased levels of Th2 cytokines at 24 h after the third challenge in PTP1B−/− mice is likely to be reflective of the very early (6 h) elevation of cytokine expression.

In summary, PTP1B deficiency in vivo unleashes early ex-
pression of a number of proinflammatory mediators, followed by rapid recruitment of effector cells from bone marrow and peripheral circulation. Remarkably, PTP1B−/− leukocytes exhibit increased activation, chemokinesis, and chemotaxis and are capable of en-
tering the tissues in significant numbers in a matter of minutes rather than hours or days after allergen exposure. In contrast, there is no change in the level of resident inflammatory cells in the nonchallenged lungs of PTP1B-deficient mice. Thus, PTP1B functions to limit induction of allergic inflammatory mediators and the recruitment of leukocytes during allergic responses.

Disclosures
The authors have no financial conflicts of interest.

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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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