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Article

Regulation of Signal Transducer and Activator of Transcription Signaling by the Tyrosine Phosphatase PTP-BL

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SUMMARY

Signal Transducer and Activator of Transcription (STAT) proteins are a family of latent cytoplasmic transcription factors that are activated by tyrosine phosphorylation after cytokine stimulation. One mechanism by which STAT signaling is regulated is by dephosphorylation through the action of protein tyrosine phosphatases (PTP). We have identified PTP-Basophil like (PTP-BL) as a STAT PTP. PTP-BL dephosphorylates STAT proteins in vitro and in vivo, resulting in attenuation of STAT-mediated gene activation. In $CD4^+$ T cells, PTP-BL deficiency leads to increased and prolonged activation of STAT4 and STAT6, and consequently enhanced T helper 1 (Th1) and Th2 cell differentiation. Taken together, our findings demonstrate that PTP-BL is a physiologically important negative regulator of the STAT signaling pathway.

INTRODUCTION

Cytokines are important regulators of immune responses, and numerous cytokines mediate their biologic function through activation of the signal transducer and activator of transcription (STAT) signaling pathway (O'Shea et al., 2002). Ligand binding to the cytokine receptor activates receptor-associated Janus kinases (JAKs), which ultimately results in the tyrosine phosphorylation of latent cytoplasmic STAT proteins and their subsequent translocation into the nucleus where they bind to target genes and effect gene transcription. For example, IL-12 and IL-4 are the cytokines that are principally responsible for activating the genetic programs necessary for the differentiation of naive $CD4^+$ T helper (Th) cells into Th1 and Th2 cells, and they do so by activating STAT4 and STAT6, respectively (Murphy and Reiner, 2002). Indeed, studies on STAT4- and STAT6-deficient mice confirmed the importance of these signaling molecules for the differentiation of Th1 and Th2 cells (Kaplan et al., 1996a, 1996b; Shimoda et al., 1996; Takeda et al., 1996; Thierfelder et al., 1996; Wurster et al., 2000).

A number of proteins have been reported to modulate STAT signaling at various steps in the signaling pathway (Shuai, 2000). In particular, efficient attenuation of STAT signaling is important for the regulation of cytokine-mediated immune responses, and recently, four main classes of regulatory molecules that negatively affect the STAT signaling pathway have been reported (Shuai and Liu, 2003). First, suppressor of cytokine signaling (SOCS) proteins bind either to activated JAK proteins resulting in the direct inhibition of JAK activity, or in some cases to the docking site in the cytokine receptor to which the STATs must bind for their activation by JAK kinases (Alexander and Hilton, 2004). Second, protein inhibitor of activated STAT (PIAS) molecules interact directly with STAT proteins and inhibit STAT function by either blocking their DNA binding activity (Chung et al., 1997; Liu et al., 1998, 2004), recruiting corepressor molecules (Arora et al., 2003) or promoting the conjugation of small ubiquitin-related modifier (SUMO) (Rogers et al., 2003; Ungureanu et al., 2003). Third, we have recently identified a LIM domain-containing protein that promotes the ubiquitination and subsequent proteosome-mediated degradation of activated STAT proteins (Tanaka et al., 2005). Finally, given that tyrosine phosphorylation of STAT proteins is required for their dimerization, nuclear translocation, nuclear retention, DNA binding activity, and transactivation, the action of protein tyrosine phosphatases (PTP) is also thought to be important in regulating the STAT signaling pathway.

Several PTP have been reported to dephosphorylate either JAK and/or STAT proteins. SHP-1 (David et al., 1995; Klingmuller et al., 1995), SHP-2 (You et al., 1999), CD45 (Irie-Sasaki et al., 2001), PTP1B (Myers et al., 2001), and TC-PTP (Simoncic et al., 2002) have all been shown to be capable of dephosphorylating activated JAK proteins. The dephosphorylation of STAT proteins can be regulated by PTP in either the cytoplasm or nucleus, and individual PTP show some degree of specificity

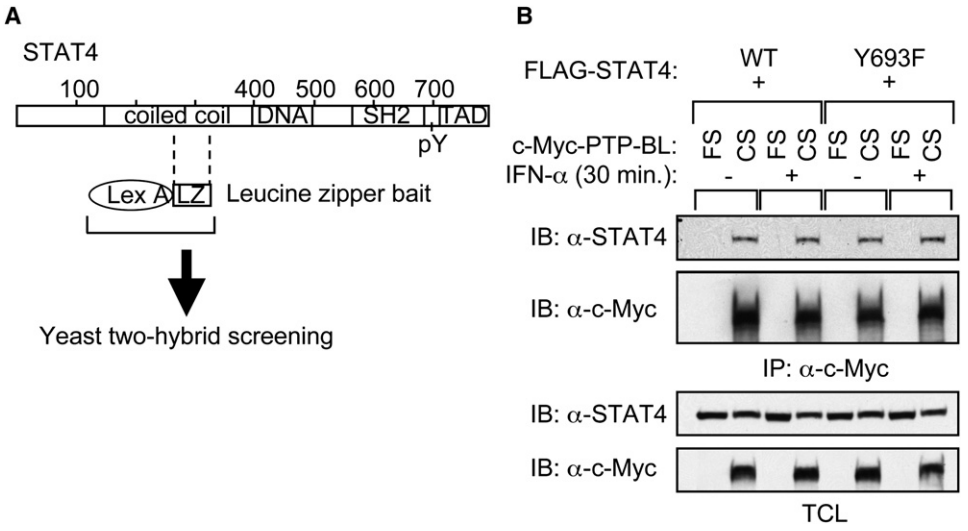

Figure 1. PTP-BL Interacts with STAT4

(A) Schematic diagram of the leucine zipper motif of STAT4 used as bait in a yeast two-hybrid screen for STAT-interacting proteins.

(B) PTP-BL interacts with STAT4 independent of tyrosine phosphorylation. Expression vectors for FLAG-tagged STAT4 wild-type (WT) or Y693F mutant (1.0 μg each) were cotransfected into 293T cells together with those for c-Myc-tagged PTP-BL frame-shift (FS) or C2285S (CS) mutants (11 μg each) as indicated. Total cell lysates prepared from the transfected cells stimulated with IFN-α (1000 U/ml) for 30 min, or left unstimulated, were immunoprecipitated (IP) with anti-c-Myc and subjected to immunoblot with anti-STAT4. Data are representative of three independent experiments.

for STAT family members. For example, SHP-2 has been shown to interact with STAT5 (Chen et al., 2003; Chughtai et al., 2002) and STAT1 (Wu et al., 2002), and SHP-2-deficient mouse embryonic fibroblasts (MEFs) have impaired dephosphorylation of STAT5 mainly in the cytoplasm and of STAT1 in nucleus, indicating that SHP-2 is involved in both cytoplasmic and nuclear dephosphorylation of STAT proteins. In contrast to SHP-2, TC-PTP acts on STAT1, and to a lesser degree STAT3, but not STAT5 or STAT6 (Meyer et al., 2003; ten Hoeve et al., 2002). In TC-PTP-deficient MEFs, both cytoplasmic and nuclear phosphorylation of STAT1 is prolonged compared to wild-type MEFs. Of the PTP that are known to act on STAT proteins, none have been shown to be primarily responsible for dephosphorylating STAT4 or STAT6. Therefore, the identification of additional PTP, in particular those that inactivate these two STAT proteins, is necessary to more fully understand the mechanism by which the STAT signaling pathway is regulated during the differentiation of Th cells.

In the present study, we used a yeast two-hybrid screen to discover molecules that interact with STAT4 in the hopes of identifying new regulators of this signaling pathway. Here we describe the interaction between STAT proteins and the tyrosine phosphatase PTP-BL, and we provide evidence that PTP-BL is a physiologically important negative regulator of the STAT signaling pathway. PTP-BL dephosphorylates not only STAT4 but also other STAT family members both in vitro and in vivo. Moreover, STAT phosphorylation is both increased and prolonged in PTP-BL-deficient cells, which results in enhanced Th1 and Th2 cell differentiation.

RESULTS

PTP-BL Interacts with STAT4

STAT4 is one of seven mammalian STAT proteins and is required for the differentiation of Th1 cells from naive $CD4^+$ T cells. Aside from the action of PIASx, which was reported to inhibit STAT4-mediated transcription by functioning as a corepressor (Arora et al., 2003), little is known about how the activity of this particular STAT protein is regulated. Thus, we sought to identify proteins that interact with STAT4 and modulate its function to further understand the regulatory mechanisms underlying STAT4 signaling. Three-dimensional structure analysis of the STAT protein showed that the N-terminal region contains a coiled-coil domain thought important for mediating protein-protein interactions (Becker et al., 1998). Indeed, this domain has been shown to interact with several transcription factors including IRF-9, c-Jun (Zhang et al., 1999), and STAT3-interacting protein (StIP1) (Collum et al., 2000). Moreover, amino acids 250 to 310 in the STAT4 coiled-coil domain encode a leucine zipper motif (Yamamoto et al., 1994). We therefore made a bait construct containing the leucine zipper region of STAT4 for use in a yeast two-hybrid screen for STAT4-interacting proteins (Figure 1A). We screened a cDNA library prepared from a mouse Th1 cell clone, and seven cDNAs were isolated from 3×10^6 independent yeast transformants. One cDNA encoded a portion of a previously identified PTP, PTP-Basophil (BAS)-like or PTP-BL (Erdmann, 2003).

To confirm the interaction between PTP-BL and STAT4 in mammalian cells, we performed a coimmunoprecipitation experiment. It is difficult to demonstrate the

interaction between a catalytically active PTP and its substrate because of the decreased affinity of the interaction after dephosphorylation of the substrate, so c-Myc-tagged expression vectors of PTP-BL encoding either a C2285S (CS) mutant that destroys PTPase activity or a frame shift (FS) mutant as control were cotransfected with a FLAG-tagged expression vector encoding wild-type (WT) STAT4 into 293T cells. 2 days after transfection, cells were stimulated with IFN-α for 30 min or left unstimulated, and total cell lysates were immunoprecipitated with c-Myc antibody and analyzed by immunoblot with anti-STAT4. As shown in Figure 1B, STAT4 could be coimmunoprecipitated with PTP-BL (CS) independent of cytokine stimulation. When the Y693F mutant of STAT4, which cannot be tyrosine phosphorylated upon cytokine stimulation, was used instead of WT STAT4, this mutant could also be coimmunoprecipitated with PTP-BL. These results demonstrate that PTP-BL interacts with STAT4, and it does so independent of phosphorylation of the conserved tyrosine residue in the C terminus of STAT4.

PTP-BL Dephosphorylates STAT4 through Its PTPase Domain

To examine whether PTP-BL overexpression can affect the phosphorylation state of STAT4 in vivo, a FLAG-tagged STAT4 expression vector was cotransfected with three forms of c-Myc-tagged PTP-BL expression vectors (frame shift mutant, FS; wild-type, WT; C-terminal PTPase domain-deleted, ΔC) into 293T cells. 24 hr after transfection, cells were stimulated with IFN-α for 30 min, or left unstimulated, and total cell lysates were immunoprecipitated with FLAG antibody and analyzed by immunoblot with anti-phospho-STAT4. In the absence of PTP-BL expression, IFN-α stimulation induced the phosphorylation of STAT4, whereas overexpression of PTP-BL (WT) resulted in a substantial decrease (up to 30%) in the amount of STAT4 phosphorylation (Figure 2A). In contrast, overexpression of mutated PTP-BL (ΔC) led to enhanced (approximately 2-fold) phosphorylation of STAT4, suggesting that mutated PTP-BL (ΔC) may act as a dominant negative in vivo.

Because tyrosine phosphorylation of STAT4 is required for it to translocate into the nucleus and activate gene transcription, the effect of PTP-BL overexpression on STAT4-mediated transactivation was investigated. 293T cells were transiently transfected with a luciferase reporter gene containing two copies of a high-affinity STAT binding site derived from IRF-1 promoter. Cotransfection of the reporter gene with a STAT4 expression vector resulted in a 3-fold increase in luciferase activity when the cells were stimulated with IFN-α (Figure 2B). Overexpression of PTP-BL (WT) led to an inhibition in STAT4-mediated transactivation from this reporter construct, whereas overexpression of mutated PTP-BL (ΔC) led to a slight increase in reporter activity. Taken together, these results suggest that PTP-BL negatively regulates the STAT4 signaling pathway by potentiating the desphosphrylation of STAT4 through its PTPase domain.

To confirm that PTP-BL can directly dephosphorylate STAT4, we performed an in vitro PTPase assay with recombinant protein. Baculovirus encoding GST-tagged PTPase domain were generated, and GST-tagged protein was obtained by affinity purification (Figure 2C). For the substrate, tyrosine phosphorylated STAT4 was immunoprecipitated from IFN-α-stimulated 293T transfectants. As shown in Figure 2D, incubation of activated STAT4 immunoprecipitates with recombinant PTPase domain resulted in complete dephosphorylation of STAT4. This result demonstrates that STAT4 is a direct substrate of PTP-BL.

PTP-BL Inhibits IL-12 Signaling at the Level of STAT4 Phosphorylation

To examine the effect of PTP-BL overexpression on IL-12R signaling, we stably transfected the Th1-like cell line 2D6 with PTP-BL (FS) or (WT) expression vectors (Figure 3A). Immunoblot analysis showed no significant differences in the overall pattern or amount of tyrosine phosphorylation after IL-12 stimulation in each of the stable cell lines (Figure 3B). In contrast, the amount of STAT4 phosphorylation was specifically decreased in 2D6 cells transfected with PTP-BL (WT) as compared to those transfected with PTP-BL (FS) after IL-12 stimulation at each time point examined (Figure 3C). After the binding of IL-12 to its receptor, the receptor-associated JAK kinase Tyk2 becomes tyrosine phosphorylated prior to its phosphorylation of STAT4 (Karaghiosoff et al., 2000; Shimoda et al., 2000). Not surprisingly, treatment of 2D6 cells with a Tyk2 inhibitor was shown to result in decreased tyrosine phosphorylation of STAT4 (Sugimoto et al., 2003). Thus, to assess the substrate specificity of PTP-BL, we examined Tyk2 phosphorylation in 2D6 transfectants. As shown in Figure 3D, 2D6 cells expressing PTP-BL (WT) have comparable amounts of Tyk2 tyrosine phosphorylation after IL-12 stimulation as that seen in cells transfected with PTP-BL (FS). These results suggest that PTP-BL acts to inhibit the tyrosine phosphorylation of STAT4 directly rather than by acting at the level of Tyk2 after IL-12 stimulation.

Finally, we examined whether PTP-BL overexpression can inhibit the expression of an endogenous STAT4-target gene. As shown in Figure 3E, IL-12-induced IFN-γ production was markedly impaired in 2D6 cells that overexpress PTP-BL, whereas PMA plus ionomycin-induced IFN-γ production was not affected in these cells. Taken together, these results suggest that PTP-BL acts directly on STAT4 to inhibit STAT4-mediated signal transduction.

PTP-BL Dephosphorylates Multiple STAT Family Members

We next examined the specificity of PTP-BL for another STAT family member, STAT6. 293T cells were cotransfected with expression vectors for FLAG-STAT6 and the three forms of c-Myc-tagged PTP-BL as in Figure 2A. Transfected cells were stimulated with IL-4, and total cell lysates were immunoprecipitated with FLAG antibody followed by immunoblot with anti-phospho STAT6. Like

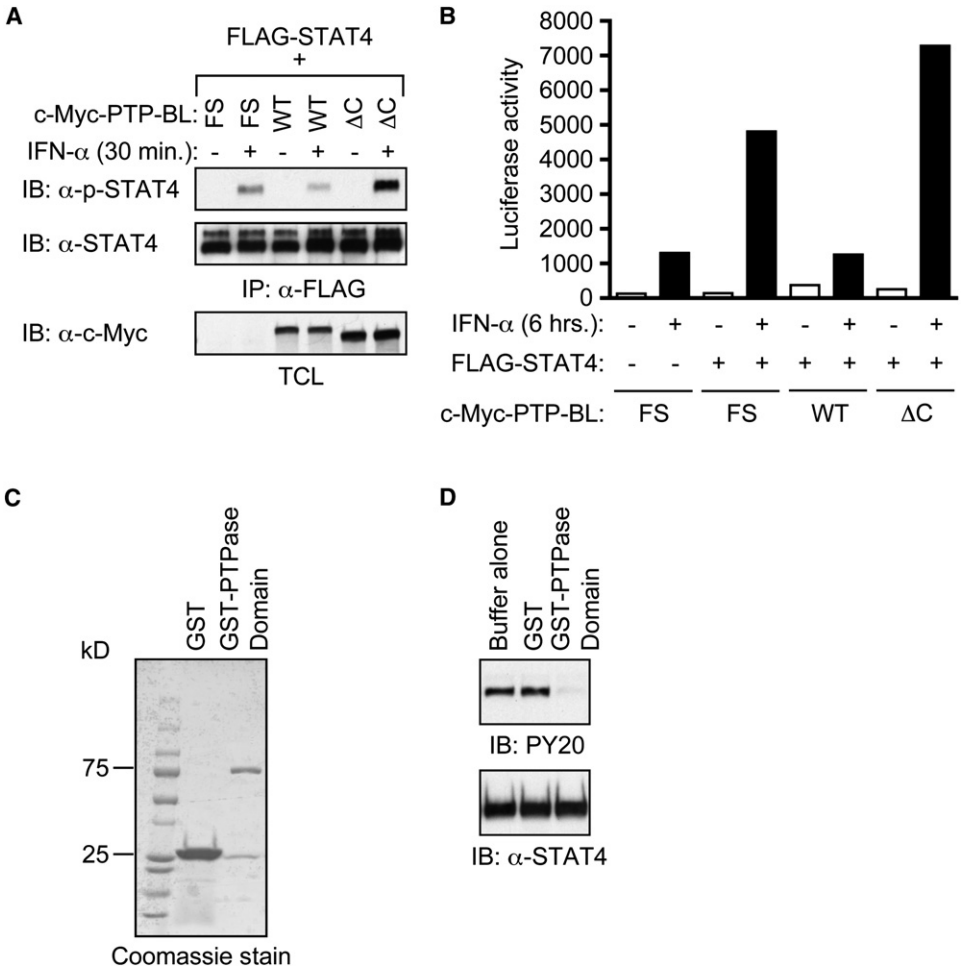

Figure 2. PTP-BL Dephosphorylates STAT4 through Its PTPase Domain

(A) PTP-BL impairs STAT4 phosphorylation. An expression vector for FLAG-tagged STAT4 (0.6 μg) was cotransfected into 293T cells together with those for c-Myc-tagged PTP-BL (FS, WT, or ΔC) (12 μg each). Total cell lysates from cells treated with IFN-α (1000 U/ml) for 30 min were immunoprecipitated with anti-FLAG and immunobloted with anti-phospho STAT4. Data are representative of three independent experiments.

(B) PTP-BL inhibits STAT4-mediated transactivation. An IRF-1 luciferase reporter construct (0.2 μg) and an expression vector for FLAG-tagged STAT4 (0.2 μg) were cotransfected into 293T cells together with those for c-Myc-tagged PTP-BL (FS, WT, or ΔC) (2.0 μg each). Cells were stimulated with IFN-α (1000 U/ml) for 6 hr and cell lysates were subjected to luciferase assay. Data are representative of three independent experiments.

(C) Purification of GST-tagged PTPase domain of PTP-BL. HighFive insect cells were infected with recombinant baculovirus that encodes either glutathoine S-transferase (GST) or GST-tagged PTPase domain of PTP-BL for 40 hr. GST-tagged proteins were prepared by affinity purification and visualized by coomassie staining.

(D) PTP-BL can directly dephosphorylate STAT4. Phosphorylated STAT4 was immunoprecipitated with anti-FLAG from IFN-α-stimulated 293T transfectants and incubated with 0.2 μg/μl recombinant proteins. Immunoprecipitates were subjected to immunoblot with anti-phospho-tyrosine (PY20). Equal loading was verified by reprobing with anti-STAT4. Data are representative of three independent experiments.

that seen with STAT4, coexpression of FLAG-STAT6 with PTP-BL (WT) resulted in a decreased amount of IL-4-induced STAT6 tyrosine phosphorylation (Figure 4A). Similar results were obtained with STAT1, STAT3, and STAT5 (see Figure S1 in the Supplemental Data available online). When the effect of PTP-BL overexpression on STAT6-mediated transactivation was assessed by luciferase assay, PTP-BL (WT) was found to inhibit IL-4-induced STAT6-mediated reporter gene activation in a dose-dependent manner (Figure 4B). Moreover, incubation of activated STAT6 immunoprecipitates with recombinant PTPase domain resulted in complete dephosphorylation of STAT6 (Figure 4C). Finally, PTP-BL overexpression had no affect on NF-κB p65-mediated transactivation (Figure 4D). Taken together, these results demonstrate that PTP-BL dephosphorylates multiple STAT proteins leading to an inhibition of STAT-mediated gene activation.

Generation of PTP-BL-Deficient Mice and PTP-BL Expression in CD4$^+$ T Cells

To further study the functional roles of PTP-BL in regulating STAT signaling, we generated PTP-BL-deficient

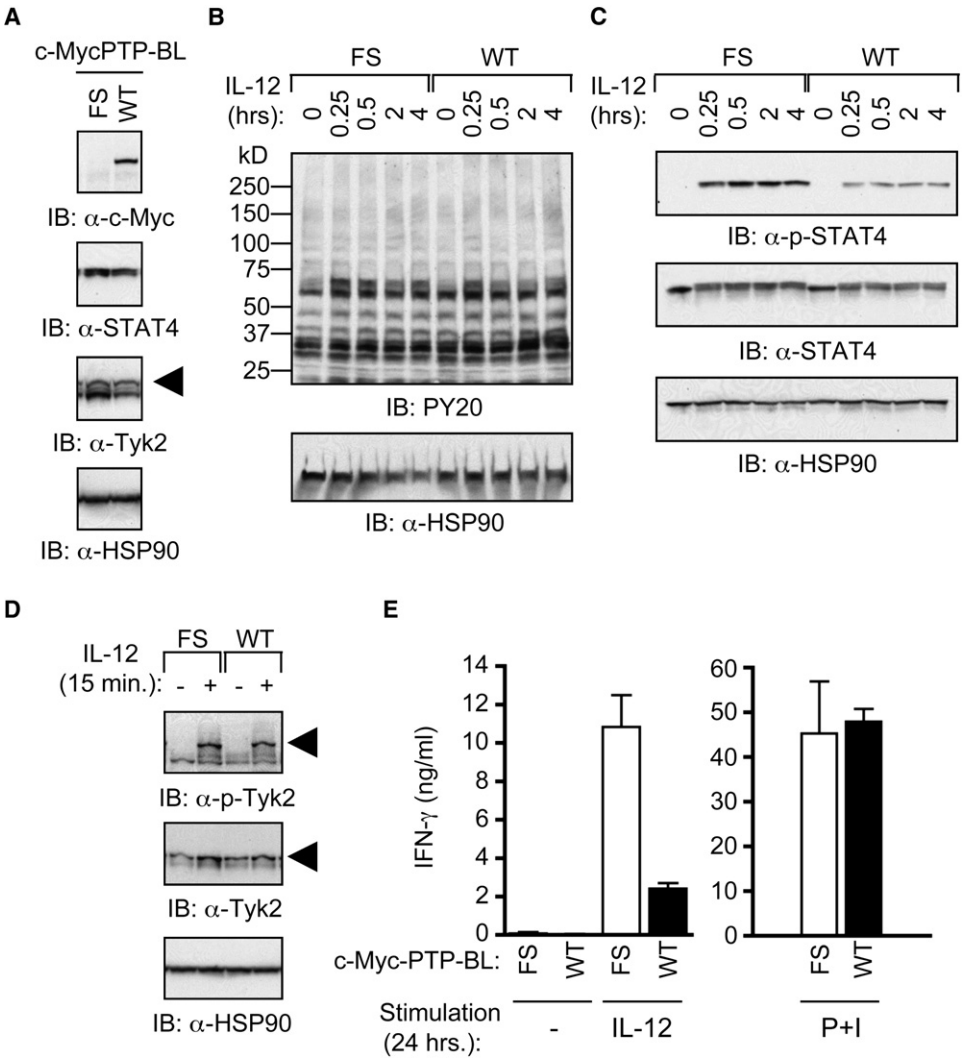

Figure 3. PTP-BL Inhibits IL-12 Signaling at the Level of STAT4 Phosphorylation

(A) Overexpression of PTP-BL in 2D6 cells.

(B) The pattern of tyrosine phosphorylated proteins in 2D6 cells overexpressing PTP-BL.

(C) PTP-BL impairs IL-12-induced STAT4 phosphorylation. IL-12-starved 2D6 cell transfectants expressing c-Myc-PTP-BL (FS or WT) were stimulated with IL-12 (1 ng/ml) for the indicated times. Total cell lysates were immunoblotted with anti-phospho STAT4. Data are representative of three independent experiments.

(D) PTP-BL does not impair IL-12-induced Tyk2 phosphorylation. IL-12-starved 2D6 transfectants were stimulated with IL-12 (1 ng/ml) for 15 min. Total cell lysates were immunoblotted with anti-phospho Tyk2. Data are representative of three independent experiments.

(E) PTP-BL overexpression impairs IL-12-induced IFN-γ production. IL-12-starved 2D6 cell clones (2×10^5 cells/well) were cultured without or with IL-12 (5 ng/ml) or PMA (50 ng/ml) plus ionomycin (1 μg/ml) in 96-well culture plates. After 24 hr, supernatants were harvested and IFN-γ production was measured by ELISA. Data are presented as mean ± SD of triplicate samples.

mice by homologous recombination in ES cells (Figure 5A). Disruption of the gene encoding PTP-BL, *Ptpn13,* was confirmed by Southern blot analysis (Figure 5B). We confirmed the absence of *Ptpn13* mRNA by northern blot analysis of total RNA isolated from kidneys of PTP-BL-deficient mice (Figure 5C). We also verified the absence of PTP-BL protein in freshly isolated PTP-BL-deficient $CD4^+$ T cells by immunoblot (Figure 5D). Flow cytometry revealed that PTP-BL-deficient mice have normal numbers of splenic lymphocyte subsets including $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, dendritic cells, and NK cells and a normal ratio of $CD4^+/CD8^+$ cells in the thymus (Figure S2A). In PTP-BL-deficient $CD4^+$ T cells, the expression of activation markers (CD69, CD62L, and CD25) both before and after anti-CD3 stimulation was comparable with WT $CD4^+$ T cells (Figure S2B). Finally, the proliferation of PTP-BL-deficient $CD4^+$ T cells after anti-CD3, IL-2, or IL-4 stimulation was also no different from WT cells (Figure S2C).

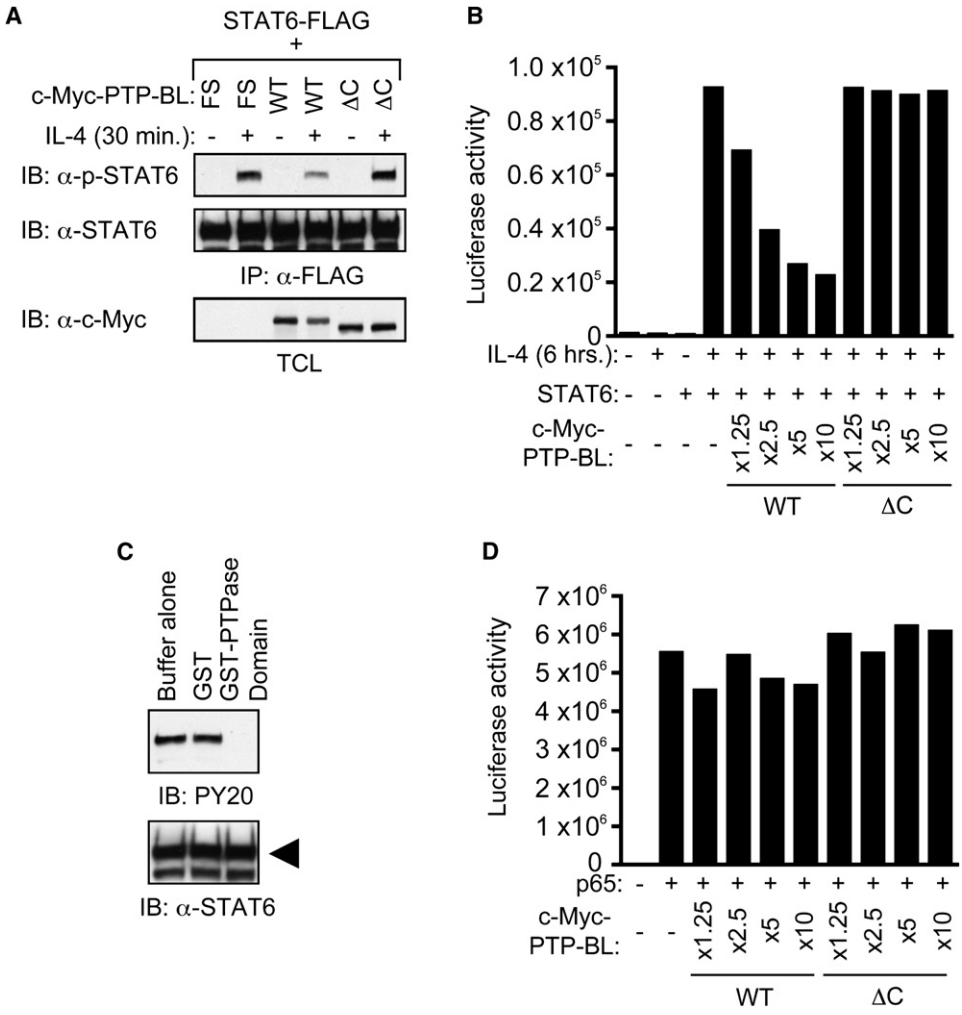

Figure 4. PTP-BL Dephosphorylates Multiple STAT Family Members

(A) PTP-BL impairs STAT6 phosphorylation. An expression vector for FLAG-tagged STAT6 (0.6 μg) was cotransfected into 293T cells together with those for c-Myc-tagged PTP-BL (FS, WT, or ΔC) (12 μg each). Total cell lysates from cells treated with IL-4 (10 ng/ml) for 30 min were immunoprecipitated with anti-FLAG and subjected to immunoblot with anti-phospho STAT6. Data are representative of three independent experiments.

(B) PTP-BL inhibits STAT6-mediated transactivation. A STAT6-responsive luciferase reporter construct (TPU474) (0.2 μg), and a STAT6 expression vector (0.2 μg) were cotransfected into 293T cells together with increasing amounts (0.25, 0.5, 1.0, and 2.0 μg) of c-Myc-tagged PTP-BL expression vectors (WT or ΔC). The total amount of transfected PTP-BL vector in each sample was adjusted to 2.0 μg by the addition of PTP-BL (FS). 24 hr after transfection, cells were stimulated with IL-4 (10 ng/ml) for 6 hr and lysates were subjected to luciferase assay. Data are representative of two independent experiments.

(C) PTP-BL can dephosphorylate STAT6 in vitro. Phosphorylated STAT6 was immunoprecipitated with anti-FLAG from IL-4-stimulated 293T transfectants and incubated with 0.25 μg/μl recombinant proteins. Immunoprecipitates were probed with anti-phospho tyrosine (PY20). Equal loading was verified by reprobing with anti-STAT6. Data are representative of two independent experiments.

(D) PTP-BL does not inhibit NF-κB-mediated transactivation. A luciferase reporter construct that contains five copies of NF-κB binding sequence (0.2 μg) and an NF-κB p65 expression vector (0.2 μg) were cotransfected into 293T cells together with c-Myc-tagged PTP-BL expression vectors (WT or ΔC). The total amount of transfected PTP-BL vector in each sample was adjusted to 2.0 μg by the addition of PTP-BL (FS). 30 hr after transfection, cells were harvested and lysates were subjected to luciferase assay. Data are representative of two independent experiments.

At the subcellular level, PTP-BL expression in both Th1 and Th2 cells was observed mainly in the cytoplasm and to a lesser extent in nuclei (Figure 5E). A similar distribution of PTP-BL in both cytoplasm and nuclei was also seen in the Th2 cell clone D10 and the Th1 cell clone 2D6 (data not shown), suggesting that PTP-BL may affect the tyrosine phosphorylation state of STAT molecules in both cellular compartments of $CD4^+$ T cells.

The Effect of PTP-BL Deficiency on STAT Activation in $CD4^+$ T Cells

As shown in Figures 2 and 3, PTP-BL overexpression reduced STAT4 phosphorylation in vivo and in vitro. Therefore, we examined the effect of IL-12 stimulation on STAT4 phosphorylation in PTP-BL-deficient $CD4^+$ T cells. Because previous studies showed that IL-12R is not expressed on resting T cells, but rather only after

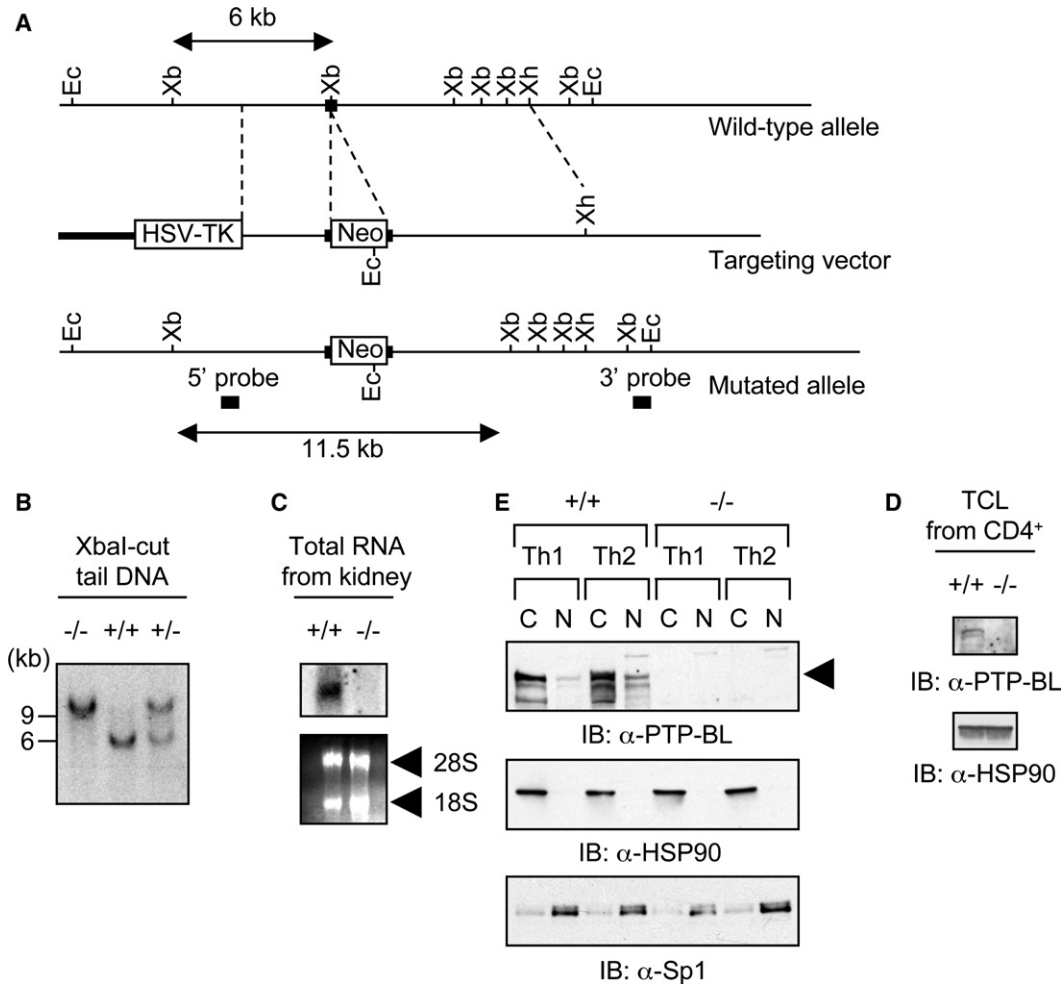

Figure 5. Generation of PTP-BL-Deficient Mice and PTP-BL Expression in CD4$^+$ T Cells

(A) Gene targeting construct for disruption of *Ptpn13*. Restriction enzymes are the following: Ec, EcoRI; Xb, XbaI; Xh, XhoI.

(B) Southern blot analysis of tail DNA from wild-type (+/+), heterozygous (+/−), and PTP-BL-deficient (−/−) mice.

(C) Northern blot analysis of total RNA isolated from the kidneys of wild-type or PTP-BL-deficient mice. 18S and 28S RNAs are shown to verify equal loading in each lane.

(D) PTP-BL expression in CD4$^+$ T cells from wild-type and PTP-BL-deficient mice. Total cell lysates were prepared from freshly isolated CD4$^+$ T cells and subjected to immunoblot with anti-PTP-BL.

(E) Subcellular localization of PTP-BL. CD4$^+$ T cells from wild-type and PTP-BL-deficient mice were differentiated under Th1 or Th2 cell conditions for 5 days. Cytoplasmic and nuclear extracts were prepared and analyzed by immunoblot with anti-PTP-BL.

TCR triggering (Nakahira et al., 2001), we stimulated CD4$^+$ T cells purified from WT and PTP-BL-deficient mice with anti-CD3, anti-CD28, and IL-12. TCR triggering plus IL-12 stimulation resulted in the phosphorylation of STAT4 in both cytoplasmic and nuclear compartments of WT CD4$^+$ T cells (Figure 6A). In accord with the subcellular localization pattern of PTP-BL seen in Figure 5D, increased STAT4 phosphorylation was observed in both subcellular compartments prepared from PTP-BL-deficient CD4$^+$ T cells, with the nuclear compartment showing an approximately 4-fold increase in the level of STAT4 phosphorylation (Figure 6A, Figure S3A). In addition, the total level of STAT4 protein in the nucleus was also increased in PTP-BL-deficient CD4$^+$ T cells after IL-12 stimulation, consistent with its increased phosphorylation status.

The balance of phosphorylation and dephosphorylation activities determines the total amount of phosphorylated STAT4 protein. If PTP-BL deficiency leads to an increase in the amount of phosphorylated STAT4 protein because of a decrease in dephosphorylation activity, we would expect to see prolonged phosphorylation of STAT4 after cytokine stimulation. We evaluated this possibility by performing a staurosporine chase experiment. CD4$^+$ T cells were stimulated with IFN-α to activate STAT4 (Nguyen et al., 2002) and then treated with staurosporine, a protein kinase inhibitor, to inhibit further STAT phosphorylation by JAK kinases. Cytoplasmic and nuclear extracts were prepared from cells harvested at various time points and subjected to immunoblot analysis to monitor the phosphorylation state of STAT4. As shown in Figure 6B (left),

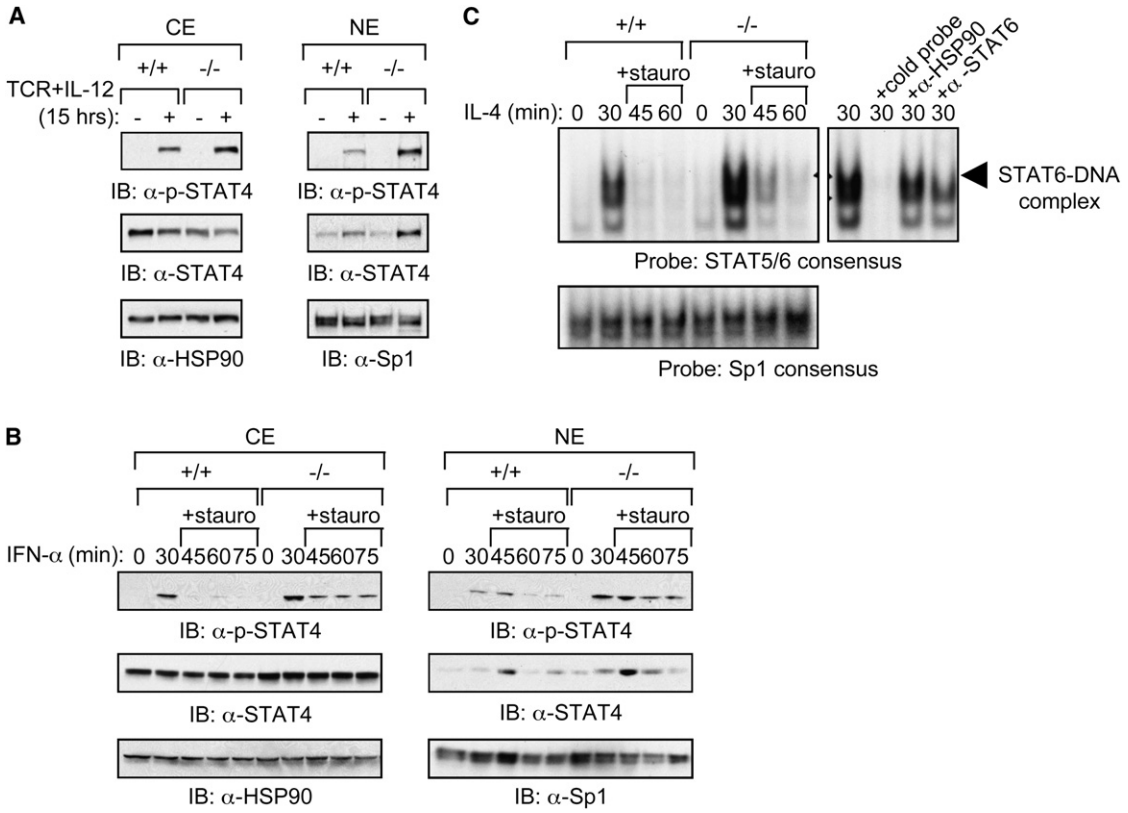

Figure 6. STAT Activation Is Increased and Prolonged in PTP-BL-Deficient CD4$^+$ T Cells

(A) STAT4 phosphorylation is increased in PTP-BL-deficient CD4$^+$ T cells. Freshly isolated CD4$^+$ T cells were stimulated with immobilized anti-CD3 (1 μg/ml for coating), 1 μg/ml anti-CD28, 100 U/ml human IL-2, 5 ng/ml murine IL-12, and 10 μg/ml anti-IL-4 for 15 hr. Cytoplasmic and nuclear extracts were subjected to immunoblot as indicated. Data are representative of three independent experiments.

(B) STAT4 phosphorylation is prolonged in PTP-BL-deficient CD4$^+$ T cells. Freshly isolated CD4$^+$ T cells were stimulated with IFN-α (3000 U/ml) for 30 min, followed by staurosporine chase (0.5 μM) for another 15, 30, or 45 min. Cytoplasmic and nuclear extracts were subjected to immunoblot as indicated. Data are representative of three independent experiments.

(C) Prolonged STAT6 DNA binding activity in nuclei of PTP-BL-deficient CD4$^+$ T cells. Freshly isolated WT and PTP-BL-deficient CD4$^+$ T cells were stimulated with IL-4 (40 ng/ml) for 30 min, followed by staurosporine chase (0.5 μM) for another 15 or 30 min. Nuclear extracts (1.3 μg) were subjected to EMSA with STAT6 and Sp1 consensus probes. Data are representative of two independent experiments.

STAT4 phosphorylation disappeared rapidly in the cytoplasmic compartment of WT cells after staurosporine treatment, while in contrast PTP-BL-deficient cells showed prolonged STAT4 phosphorylation even 45 min after the addition of staurosporine. We performed a densitometric analysis to quantify the amount of phosphorylated STAT4 protein present in the cytoplasmic compartment after staurosporine treatment (Figure S3B, left). In WT cells, the amount of phosphorylated STAT4 reached background amounts by 45 min after the addition of staurosporine, whereas PTP-BL-deficient cells still retained approximately 40% of their maximum amount of phosphorylated STAT4 at that time point. In the nuclei of WT cells, STAT4 phosphorylation reached background amounts 30 min after the addition of staurosporine, whereas STAT4 phosphorylation was at peak levels at the same time point PTP-BL-deficient cells (Figure S3B, right). In accord with the prolonged phosphorylation of STAT4 seen in PTP-BL-deficient nuclei, a concomitant increase in the amount of total STAT4 protein was also observed (Figure 6B). Similar results were obtained when STAT1 and STAT5 phosphorylation were examined by staurosporine chase (data not shown).

We next examined whether IL-4-induced STAT6 phosphorylation is similarly prolonged in PTP-BL-deficient CD4$^+$ T cells. However, we found that nuclear STAT6 phosphorylation in freshly isolated CD4$^+$ T cells was too weak to detect by immunoblot analysis, and we did not observe any difference in the amounts of STAT6 phosphorylation between WT and PTP-BL-deficient Th2 cells, possibly because of the sensitivity of the antibody used (data not shown). The DNA-binding activity of STAT proteins is correlated with their amount of tyrosine phosphorylation, so we assessed STAT6 DNA-binding activity in freshly isolated WT and PTP-BL-deficient CD4$^+$ T cells by electrophoretic mobility shift assay (EMSA) (Figure 6C). In WT nuclei, IL-4-induced STAT6 DNA-binding activity vanished completely after 15 min of staurosporine chase. In contrast, STAT6 DNA-binding activity was still maintained in extracts from PTP-BL-deficient cells harvested

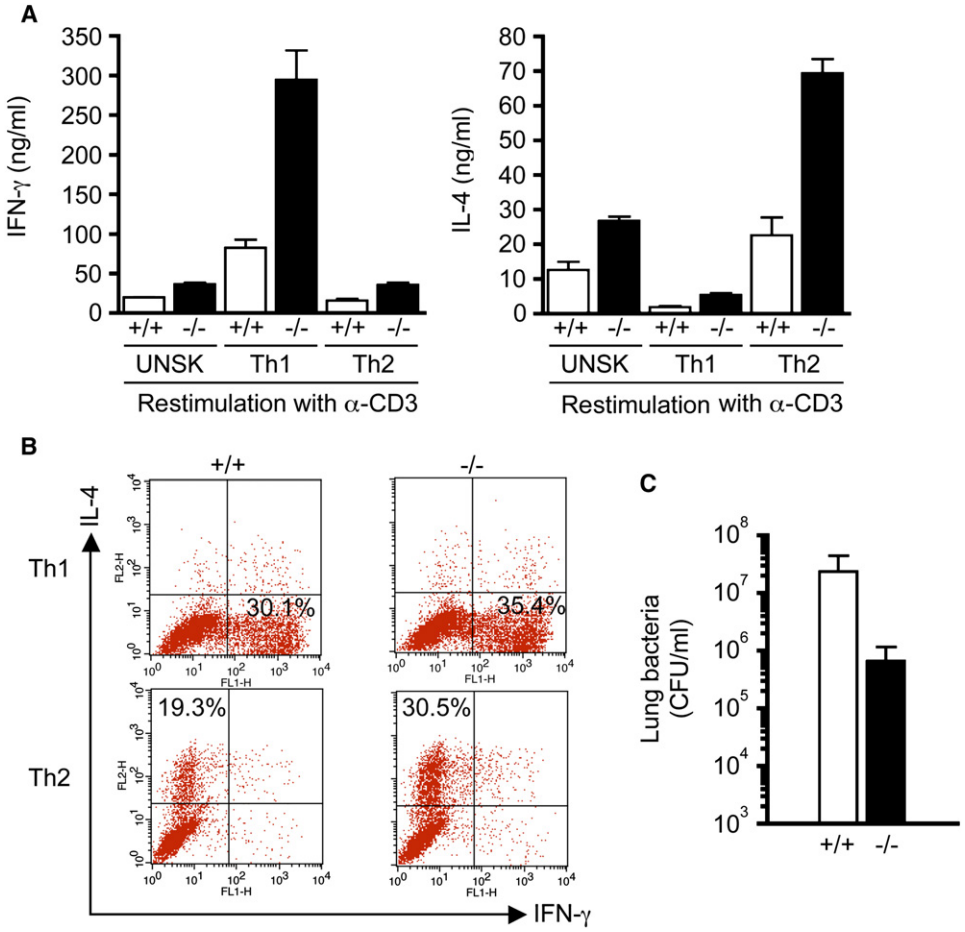

Figure 7. Enhanced Th1 and Th2 Cell Differentiation in PTP-BL-Deficient CD4$^+$ T Cells

(A) Increased IFN-γ and IL-4 production by PTP-BL-deficient Th and Th2 cells after secondary stimulation. CD4$^+$ T cells isolated from WT and PTP-BL-deficient mice were differentiated under unskewed (UNSK), Th1-, and Th2-skewing conditions for 5 days and then restimulated with plate-bound anti-CD3. IFN-γ and IL-4 production was measured by ELISA. Data are presented as mean ± SD of triplicate samples and are representative of four independent experiments.

(B) Enhanced Th1 and Th2 cell differentiation in PTP-BL-deficient CD4$^+$ T cells. The generation of Th1 and Th2 cells was assessed by intracellular cytokine staining. The percentages of cells in each quadrant are shown. Data are representative of three independent experiments.

(C) Enhanced pulmonary bacterial clearance in PTP-BL-deficient mice. 48 hr after intratracheal *K. pneumoniae* infection (7 mice per each group), CFU/ml lung homogenate were assessed in WT and PTP-BL-deficient mice. Data are presented as mean ± SEM.

at same time point. Taken together, these results demonstrate that STAT phosphorylation is both increased and prolonged in the absence of PTP-BL and further indicate that PTP-BL is responsible for the dephosphorylation of STAT proteins in vivo.

Increased Th1 and Th2 Differentiation in PTP-BL-Deficient CD4$^+$ T Cells

Because STAT4 and STAT6 have been shown to play critical roles in the differentiation of Th cells (Murphy and Reiner, 2002), we examined the effect of PTP-BL deficiency on this process. CD4$^+$ T cells isolated from WT and PTP-BL-deficient mice were differentiated under unskewed (UNSK), Th1, or Th2 culture conditions in vitro. After restimulation with anti-CD3, ELISA of culture supernatants was performed to measure the production of IFN-γ and IL-4. Increased production of IFN-γ under Th1-skewing conditions and enhanced production of IL-4 under Th2 culture conditions was observed in PTP-BL-deficient CD4$^+$ T cells as compared to WT cells (Figure 7A). We also analyzed the status of T helper cell differentiation by intracellular cytokine staining, and enhanced Th cell differentiation was confirmed in PTP-BL-deficient CD4$^+$ T cells (Figure 7B). Thus, PTP-BL-deficient CD4$^+$ T cells have an enhanced potential to develop into Th1 and Th2 cells, which can be ascribed to increased and/or prolonged STAT4 and STAT6 activation.

Effect of PTP-BL Deficiency on Lung Host Defense

During *K. pneumoniae* pneumonia, interrupting T cell cytokine signaling impairs host defenses (Deng et al., 2004), while increasing T cell cytokine production improves

bacterial killing (Ruan et al., 2006). Because PTP-BL-deficient T cells demonstrated increased activities including cytokine elaboration, we tested whether PTP-BL deficiency could improve host defense against *K. pneumoniae* in the lungs. Bacteria multiplied in the lungs over the 2 day time period, as in prior studies (Deng et al., 2004; Ruan et al., 2006), but wild-type mice had 35-fold more living bacteria in their lungs than did PTP-BL-deficient mice (Figure 7C). These data suggest that PTP-BL deficiency results in improved host defense against intrapulmonary *K. pneumoniae*.

DISCUSSION

The STAT signaling pathway is negatively regulated by several diverse groups of molecules including families of SOCS, PIAS, and PTP proteins (Shuai and Liu, 2003). Because tyrosine phosphorylation of STAT proteins is known to be critical for their functional activity, regulating dimerization, nuclear translocation, nuclear retention, DNA binding, and transactivation, PTP have been implicated as particularly important molecules for the efficient attenuation of STAT signaling (Andrews et al., 2002; Shuai et al., 1996). To date, it has been shown that TC-PTP and SHP-2 act on STAT1-STAT5 and STAT1-STAT3, respectively (Chen et al., 2003; Chughtai et al., 2002; Meyer et al., 2003; ten Hoeve et al., 2002; Wu et al., 2002), but PTP responsible for the dephosphorylation of STAT2, STAT4, and STAT6 have not yet been identified.

In the present study, we have identified PTP-BL as a PTP that acts on multiple STAT proteins. Specifically, we demonstrated that PTP-BL and STAT4 physically interact and that several STAT proteins are substrates for the tyrosine dephosphorylation activity of PTP-BL. Furthermore, STAT4 and STAT6 phosphorylation is increased and prolonged in the cytoplasm and nuclei of PTP-BL-deficient $CD4^+$ T cells, and this correlates with increased amounts of Th cell differentiation and subsequent cytokine production. We conclude that PTP-BL is a physiologically important negative regulator of the STAT signaling pathway.

PTP-BL, and its human homolog PTP-BAS, are known to possess at least seven potential protein-protein interaction domains including a kinase noncatalytic C-lobe (KIND) domain, a Four-point-one-Ezrin-Radixin-Moesin (FERM) domain, and five PSD-95-*Drosophila* discs large-Zonula occludens (PDZ) domains, and many potential interacting proteins have been identified (Erdmann, 2003). In addition to these seven interaction domains, PTP-BL also contains a leucine zipper (LZ) domain in the N terminus of the molecule (Chida et al., 1995), and because PTP-BL was isolated from a yeast two-hybrid screening with the LZ motif of STAT4 as a bait, it is possible that these domains may be important in mediating the interaction of PTP-BL and STAT proteins.

Several proteins have been reported to be potential substrates for the enzymatic activity of PTP-BL. In cell lines transfected with PTP-BL expression vectors, or in vitro PTPase assays with recombinant PTP domain, RIL (Cuppen et al., 1998), β-Catenin (Erdmann et al., 2000), c-Src (Palmer et al., 2002), IκBα (Nakai et al., 2000), and EphirinB (Palmer et al., 2002) were shown to be capable of being dephosphorylated. However, the functional significance of PTP-BL-induced dephosphorylation of any of these targets has not clearly been established. In this study, we showed that STAT proteins are direct substrates of PTP-BL, and, by using PTP-BL-deficient $CD4^+$ T cells, we further demonstrated that PTP-BL serves as a physiologically important regulator STAT-mediated responses such as those leading to the differentiation of Th cells.

In correlation with the subcellular localization pattern of PTP-BL, the inhibitory effect of PTP-BL on STAT phosphorylation was evident in both cytoplasmic and nuclear fractions. This result indicates that PTP-BL can act as both a cytoplasmic and nuclear STAT PTP, as is the case with some other recently identified PTP. For example, prolonged phosphorylation of STAT5 was observed in the cytoplasm of Shp-2-deficient cells, and SHP-2 has also been shown to be involved in the dephosphorylation of STAT1 in the nucleus (Chen et al., 2003; Chughtai et al., 2002; Wu et al., 2002). In contrast, TC-PTP appears to act as a nuclear PTP to inactivate STAT1 and STAT3 (Meyer et al., 2003; ten Hoeve et al., 2002), while PTP1B has been implicated as a cytoplasmic PTP, acting on STAT5 (Aoki and Matsuda, 2000). Taken together with previous work on Tc-ptp- and Shp-2-deficient cells, our present study on PTP-BL demonstrates that PTP plays a significant role in attenuating STAT signaling.

A number of diverse functions have been ascribed to PTP-BL, given the varied proteins that it has been shown to interact with. For example, PTP-BL has been suggested to act as a scaffolding protein in the regulation of the cytoskeleton (Erdmann, 2003), to confer resistance to FAS-induced cell death (Ivanov et al., 2003; Sato et al., 1995), to dephosphorylate EphrinB and thus regulate various developmental processes (Palmer et al., 2002), and to be involved in the regulation of cytokinesis (Herrmann et al., 2003). Recently, independent reports on the characterization of PTP-BL-deficient mice revealed a phenotype of impairment in motor nerve repair (Wansink et al., 2004) and the involvement of PTP-BL in both retinal ganglion cell neurite initiation and survival of activated retinal glia (Lorber et al., 2005).

In this study, we have demonstrated that PTP-BL is a physiologically important phosphatase for STAT proteins; overexpression of PTP-BL leads to an attenuation of STAT signaling, while PTP-BL deficiency results in increased and prolonged STAT phosphorylation. Although PTP-BL appears to act on multiple STAT family members, it is the first tyrosine phosphatase to be shown to effectively dephosphorylate STAT4 and STAT6 in particular. To date, several molecules that impinge on the JAK-STAT signaling pathway and result in a perturbation in Th1 and Th2 cell differentiation have been identified. For example, analysis of SOCS1-deficient mice has revealed that SOCS-1 can modulate Th1 and Th2 cell development and IFN-γ and IL-4 production (Fujimoto et al., 2002).

In addition, heterozygous motheaten (me/−) mice, which express decreased amounts of SHP-1, show increased generation of Th2 cells as well as elevated STAT6 phosphorylation (Kamata et al., 2003). However, the targets of SOCS1 and SHP-1 are JAK kinases and there little is known about the regulation of the lineage decision of $CD4^+$ T cells at the level of STAT proteins. Here we have shown that increased and prolonged STAT phosphorylation after cytokine stimulation correlates with increased production of two hallmark cytokines, IFN-γ and IL-4, in PTP-BL-deficient $CD4^+$ T cells, suggesting that PTP-BL negatively regulates Th cell differentiation by inactivating STAT4 and STAT6.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Screening

The leucine zipper bait was generated by subcloning a portion of the murine STAT4 cDNA encoding amino acids 250–310 in-frame into pEG202 (Clontech). Yeast strain EGY48 was sequentially transformed with bait construct, pSH17 reporter plasmid (Clontech), and pJG4-5 (Clontech) expression vector containing a cDNA library from a mouse Th1 cell clone stimulated with anti-CD3 for 5 hr. Positive colonies were screened on dropout and X-gal plates and subjected to sequencing analysis.

Plasmid Constructs and Reagents

FLAG-tagged murine STAT1, STAT4 (WT and Y693F), and STAT6 were cloned into pcDNA3 (Invitrogen). c-Myc tagged PTP-BL (WT, FS, CS, and ΔC) were generated in pCMV-Myc (Clontech). The frame-shift (FS) mutant was prepared by digestion of the 5-prime region of the PTP-BL cDNA with NsiI followed by self-ligation to induce a frame-shift in the reading frame. The 2× IRF-1 luciferase reporter and STAT6-responsive reporter constructs were gifts from T. Hoey. The NF-κB luciferase reporter construct and NF-κB p65 expression vector were gifts from L. Glimcher. Anti-phospho STAT4 (71-7900; Zymed Laboratory), anti-phospho STAT6 (#9361; Cell Signaling Technology), anti-phospho STAT1 (#9171; Cell Signaling Technology), anti-STAT6 (M-20) (sc-981; Santa Cruz Biotechnology), anti-STAT4 (C-20) (sc-486; Santa Cruz Biotechnology), anti-phospho STAT5 (#9351; Cell Signaling Technology), anti-STAT5 (C-17) (sc-835; Santa Cruz Biotechnology), anti-STAT1 p84/p91 (E-23) (sc-346; Santa Cruz Biotechnology), anti-phospho STAT3 (#9131, Cell Signaling Technology), anti-STAT3 (C-20) (sc-482; Santa Cruz Biotechnology), anti-phospho Tyk2 (Tyr 1054/1055) (#9321; Cell Signaling Technology), anti-Tyk2 (C-20) (sc-169; Santa Cruz Biotechnology), anti-Sp1 (PEP2) (sc-59; Santa Cruz Biotechnology), anti-HSP90 (H-114) (sc-7947; Santa Cruz Biotechnology), anti-c-Myc (9E10) (sc-40; Santa Cruz Biotechnology), anti-FLAG M2 agarose beads (A2220; Sigma), anti-phospho tyrosine antibody (PY20) (P11120; BD Transduction Laboratories), HRP-rabbit anti-mouse IgG (H+L) (61-6520; Zymed), and HRP-goat anti-rabbit IgG (H+L) (81-6120; Zymed) were purchased from the indicated companies. Anti-PTP-BL anti-serum was a gift from K. Erdmann. Staurosporine was purchased from Calbiochem (#569396). For the detection of immunoblots, Immun-Star HRP substrate kit (170-5040; BIO-RAD) or ECL western blotting detection reagent (RPN2106; Amersham) were used.

Cell Culture

293T cells were maintained in DMEM supplemented with 10% FCS. 2D6 cells were maintained in RPMI supplemented with 10% FCS in the presence of 500 pg/ml mouse IL-12.

Transient Transfections and Reporter Assays

Transfections for 293T cells were done with Effectene (301427; Qiagen). Luciferase assays were performed according to the manufacturer's protocol (Promega).

Generation of 2D6 Stable Transformants

Five million 2D6 cells were transfected with linearized pCMV-Myc-PTP-BL (3.5 μg) along with linearized pcDNA3 empty vector (0.5 μg). For transfection, Amaxa nuclofector II module (Amaxa biosystems) was used together with Cell Line Nucleofector kit V (VCA-1003; Amaxa biosystems) under the control of program X-001. Single cell clones were isolated after selection in the presence of 1 mg/ml Geneticin (118111-031; GIBCO) for 2 weeks.

Baculovirus Expression and Purification of GST-Tagged Protein

BAC-TO-BAC Baculovirus Expression System (Invitrogen) was used to generate recombinant baculovirus according to the manufacturer's protocol. In brief, glutathione S-transferase (GST) protein and GST-tagged PTPase domain were cloned into pFASTBAC1 donor plasmid. The recombinant plasmids were transformed into bacmid-carrying *E. coli* strain (DH10BAC) to obtain the recombinant bacmids by transposition in *E. coli*. The resultant recombinant bacmids were transfected into Sf9 insect cells to get the recombinant baculovirus. Recombinant proteins were purified from HighFive cells (Invitrogen) with GST bind resin (70541-3; Novagen). When lysates were prepared from HighFive cells, a 3× higher amount of Complete Mini protease inhibitor cocktail (#11836153001; Roche) than usual was used, along with 1 mM phenylmethylsulfonyl fluoride (PMSF).

PTPase Assay

In vitro PTPase assay was performed essentially as described (Erdmann et al., 2000). In brief, phosphorylated FLAG-tagged STAT4 or FLAG-tagged STAT6 were immunoprecipitated from IFN-α- or IL-4- stimulated 293T transfectants, respectively. Immunoprecipitates were incubated with GST-tagged proteins in 25 mM HEPES (pH 7.5), 5 mM EDTA, 10 mM DTT at 37°C for 3 hr and subjected to immunoblot with anti-phospho-tyrosine (PY20).

Immunoprecipitation and Immunoblot Analysis

Total cell lysates (TCLs) were prepared by lysing cells in 2× lysis buffer (50 mM Tris-HCL [pH 8.0], 300 mM NaCl, 1% TritonX-100, 2 mM Na_3VO_4, 20 mM NaF) supplemented with Complete Mini protease inhibitor cocktail. For immunoprecipitation, TCLs in 1× lysis buffer were incubated with Protein A/G agarose beads (sc-2003; Santa Cruz Biotechnology) conjugated with antibodies as indicated and subjected to SDS-PAGE followed by immunoblot analysis. For coimmunoprecipitation studies, 250 mM NaCl was used.

Generation of PTP-BL-Deficient Mice

Murine *Ptpn13* was isolated from a C57BL/6 genomic library. To disrupt *Ptpn13*, we constructed a targeting vector in which the neomycin resistance gene cassette was inserted into the exon encoding amino acids 452–526. The targeting vector was electroporated into C57BL/6 ES cells, followed by G418 selection. Colonies containing the disrupted allele were identified by Southern blot analysis. Targeted ES cell clones were injected into C57BL/6 blastocysts and implanted into pseudopregnant females to obtain chimeric mice that were then bred for germline transmission. All experiments were performed according to the guidelines of the Harvard Medical Area Standing Committee on Animals.

Preparation of Cytoplasmic and Nuclear Extracts

Cytoplasmic and nuclear extracts were prepared with hypotonic buffer (20 mM HEPES-NaOH [pH 7.9], 10 mM KCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 20 mM NaF, 0.1% Nonidet P-40, 1 mM DTT) and hypertonic buffer (20 mM HEPES-NaOH [pH 7.9], 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 20 mM NaF, 420 mM NaCl, 20% glycerol, 1 mM DTT), respectively. These buffers were supplemented with proteinase inhibitor cocktail.

EMSA

Binding reaction was performed in a total volume of 20 μl in the following buffer: 20 mM HEPES-NaOH (pH 7.9), 5 mM $MgCl_2$, 50 mM KCl,

1 mM DTT, and 6% glycerol. Each reaction, also containing 2 μg of poly (dI-dC) and 32P end-labeled probe (60,000 cpm/reaction), was initiated by the addition of 1.3 μg nuclear extract and was allowed to incubate at room temperature for 30 min before electrophoretic analysis on a 4% native PAGE in 0.5× Tris-borate-EDTA (TBE) buffer. The STAT5/6 consensus oligonucleotide probe (5′-GTATTTCCCA GAAAAGGAAC-3′; sc-2567) and Sp1 consensus oligonucleotide probe (5′-ATTCGATCGGGGCGGGGCGAGC-3′; sc-2502) were purchased from Santa Cruz Biotechnology.

In Vitro Th Cell Differentiation

Murine $CD4^+$ T cells were isolated by MACS (Miltenyi Biotec) and stimulated for 4 days with immobilized anti-CD3 (1 μg/ml for coating) in the presence of 1 μg/ml anti-CD28 (37.51; BD Pharmingen) and 100 U/ml human IL-2. For Th1 cell differentiation, murine IL-12 (5 ng/ml) and anti-IL-4 (10 μg/ml) were added to the culture media, and for Th2 cell differentiation, murine IL-4 (10 ng/ml) was added. The cells were cultured with just cytokines for another day, harvested, washed, and restimulated with immobilized anti-CD3 (0.2 μg/ml for coating). After 24 hr, culture supernatants were harvested for ELISA measurement of cytokine production.

Intracellular Cytokine Staining

Th1- or Th2-polarlized $CD4^+$ T cells were obtained as described above and restimulated with immobilized anti-CD3 (5 μg/ml for coating) for 5.5 hr in the presence of 3 nM Monensin (Sigma) for the last 2.5 hr. These cells were stained with PE-labeled anti-mouse IL-4 (#554435; BD Pharmingen), FITC-labeled anti-mouse IFN-γ (#554411; BD Pharmingen) and their isotype controls. Flow cytometric analysis was performed on a FACSCalibur (Becton Dickinson), and live cells were gated based on FSC and SSC profiles.

Bacterial Pneumonia

Mice (7-week-old C57BL/6 males) were infected by intratracheal instillation as previously described (Jones et al., 2005). After anesthesia by ketamine (50 mg/kg) and xylazine (5 mg/kg) i.p., trachea were surgically exposed and an angiocatheter was placed in the left bronchus, through which mice received 50 μl of 4×10^4 colony-forming units (CFU)/ml *Klebsiella pneumoniae* (43816 from the American Type Culture Collection; Manassas, VA). After 48 hr, mice were euthanized with an overdose of halothane. Lungs were homogenized in 10 ml sterile water, serially diluted, and plated on agar for overnight growth at 37°C. Bacterial burdens were expressed as CFU/ml lung homogenate.

Densiometric Analysis

Immunoblot signals were quantified with ImageJ (http://rsb.info.nih.gov/ij).

Supplemental Data

Three Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/26/2/163/DC1/.

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