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PTPN22 Alters the Development of Regulatory T Cells in the Thymus

Christian J. Maine,* Emma E. Hamilton-Williams,* Jocelyn Cheung,* Stephanie M. Stanford,† Nunzio Bottini, † Linda S. Wicker,‡ and Linda A. Sherman*

PTPN22 encodes a tyrosine phosphatase that inhibits Src-family kinases responsible for Ag receptor signaling in lymphocytes and is strongly linked with susceptibility to a number of autoimmune diseases. As strength of TCR signal is critical to the thymic selection of regulatory T cells (Tregs), we examined the effect of murine PTPN22 deficiency on Treg development and function. In the thymus, numbers of pre-Tregs and Tregs increased inversely with the level of PTPN22. This increase in Tregs persisted in the periphery and could play a key part in the reduced severity observed in the PTPN22-deficient mice of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. This could explain the lack of association of certain autoimmune conditions with PTPN22 risk alleles.  The Journal of Immunology, 2012, 188: 5267–5275.

PTPN22 encodes a phosphatase known as lymphoid tyrosine phosphatase (Lyp) in humans and PEST-enriched protein tyrosine phosphatase (Pep) in mice. PTPN22 is expressed in T, B, NK, and dendritic cells (1). It has been most widely studied in T cells where it functions to dephosphorylate key members of the TCR signaling pathway including ZAP70, Src family kinases such as Lck, and VAV (2, 3).

Interest has been generated in this gene due to the observation that a Lyp polymorphism, R620W, is associated with a number of human autoimmune conditions including type 1 diabetes (T1D), rheumatoid arthritis, systemic lupus erythematosus, and Graves disease (4–6). Of interest, no association or a negative association has been observed between this polymorphism and other autoimmune diseases including multiple sclerosis and Crohn’s disease (7, 8).

The functional significance of this polymorphism and the molecular basis underlying these autoimmune associations is unclear. Several studies have suggested the mutation results in a gain-of-function in the phosphatase that reduces TCR signaling, a paradoxical observation considering its association with autoimmunity (6, 9, 10). It has been hypothesized this decreased signaling may alter thymic selection thresholds leading to escape of autoreactive T cells into the periphery or decrease regulatory T cell (Treg) number and/or function. However, others have reported that the consequence of this polymorphism is a decrease in phosphatase activity and increased signaling, resulting in an altered signaling threshold in T and B lymphocytes and dendritic cells (11, 12). Recent studies in which the Pep phosphatase in mice was mutated to express the equivalent of the Lyp R620W disease-associated polymorphism suggested this results in rapid degradation of the phosphatase, thereby resulting in increased responsiveness in T cells and dendritic cells (12). These same investigators also demonstrated reduced levels of Lyp in human T cells homozygous for the risk-related allele, suggesting reduced levels of phosphatase may be associated with disease.

We hypothesized that alteration in PTPN22 activity should affect Treg development, function, and homeostasis. Changes in TCR signaling may alter the number of Tregs generated in the thymus as thymic selection is dependent on the strength of signal received by the developing thymocyte, and Tregs require greater strength of signal than conventional T cells during development (13–17). PTPN22 directly affects ZAP70 signaling, a key molecule upstream of several different pathways that could affect Foxp3 expression (3, 18). Furthermore, Treg development and homeostasis is influenced by IL-2 and other related common γ-chain cytokines (19–23). If PTPN22 expression were to influence genes upstream of these cytokines and their receptors, this could lead to increased development and homeostasis of Tregs.

In this study, we have used PTPN22-deficient mice to investigate the impact on Treg development and function of altered levels of PTPN22. We show that thymic numbers of Tregs are increased in the absence of PTPN22, a trend that continues in the periphery and results in a functional outcome in a mouse model of autoimmunity.

Materials and Methods

Mice

Experimental procedures were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. PTPN222/− mice were obtained from Dr. Andrew Chan (Genentech, San Francisco, CA) and have previously been described (24). PTPN222/− mice were obtained by interbreeding PTPN222/− mice with C57BL/6 (The Jackson Laboratory, Bar Harbor, ME). Foxp3 GFP mice were provided by Dr. Mitch Kronenberg (La Jolla Institute of Allergy and Immunology) with

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permission from Dr. Alexander Rudensky (Memorial Sloan-Kettering Cancer Center).

Experimental autoimmune encephalomyelitis induction

Female mice, at least 9 wk old, were injected s.c. with 200 μg myelin oligodendrocyte protein (MOG) 35–55 (Genscript, Piscataway, NJ) in CFA (Difco, Detroit, MI), with half of the mice injected behind the neck and the other half at the base of the tail. Two hours later, mice were injected i.p. with 300 ng pertussis toxin (List Biologicals, Campbell, CA), and another injection was given 24 h later. Mice were assessed daily for 28 d for signs of disease using the following clinical scores: 0, no disease; 1, limp tail; 2, hind limb weakness, wobbly walk; 3, hind limb paralysis; 4, front limb paralysis; 5, death/moribund.

For Treg depletion experiments, mice were also injected i.p. with 300 μg anti-CD25 Ab (PC-61) (The Scripps Research Institute [TSRI] Ab core, La Jolla, CA) on day 21 and day 4.

Flow cytometry

Cells to be stained were resuspended in FACS buffer (HBSS containing 1% FCS) and incubated with the indicated Abs for 15 min on ice. Cells were then washed in FACS buffer before acquisition on an LSR-II flow cytometer (BD Biosciences, Franklin Lakes, NJ) and analysis using FlowJo (TreeStar). Abs used were anti-mouse CD4 FITC and PerCP (both BD Pharmingen, San Diego, CA), CD8 Pacific blue/PerCP (both BioLegend, San Diego, CA), CD25 allophycocyanin and FITC, GITR–biotin, and CD122–biotin (all BioLegend). Biotinylated Abs were detected with streptavidin–FITC or PerCP (BD Biosciences). For intracellular staining of Treg markers, an intracellular staining kit (Fix/Perm, eBioscience, San Diego CA) was used together with anti-mouse Foxp3 PE (eBioscience), Helios Pacific blue (BioLegend), and CTLA-4 allophycocyanin (BioLegend). For cell sorting, splenocyte and thymocyte single-cell suspensions were Ab labeled as described earlier, and the desired populations were sorted using FACSaria (BD Biosciences) or Mo Flo XDP (Beckman Coulter, Indianapolis, IN) by the TSRI flow cytometry core facility.

IL-2 stimulation and p-STAT5 staining

FACS-sorted CD4+CD8− T cells from spleens were incubated in serum-free RPMI 1640 (Invitrogen, Grand Island, NY) for 30 min at 37˚C. Cells were then washed and resuspended in complete RPMI 1640 (cRPMI) with 20 ng/ml IL-2 (kindly provided by Dr. Charles Surh, TSRI) for 0, 2, 5, and 20 min at 37˚C. Cells were immediately fixed using 2% v/v paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and incubated at room temperature for 10 min. Cells were then resuspended in 90% ice-cold methanol (Sigma Aldrich, St. Louis, MO) and incubated at −20˚C for 30 min. Cells were then surface stained, and p-STAT5 was stained using anti-human p-STAT5 allophycocyanin (BD Pharmingen).

Calcium flux

Thymocytes were rested in cRPMI for 20 min at 37˚C before staining. Thymocytes from one genotype (5 × 10⁶) were labeled with 1 μg Cy5 dye.
in cRPMI (GE Healthcare, Chalfont, U.K.) for 5 min at room temperature, and the other genotype was left unstained. Cy5-labeled cells were washed and mixed with unlabeled cells. These cells were then loaded with INDO-1 AM (Invitrogen) in cRPMI media for 30 min at 37˚C. After washing, the cells were stained with CD4 PerCP (BD Pharmingen), CD8 PeCy7 (BD Pharmingen), and CD3 biotin (eBioscience) and resuspended in HBSS (Life Technologies) and kept on ice. Cells were then warmed to 37˚C prior to running the sample, which then ran for 30 s to establish a baseline, and 10 µg/ml streptavidin–PE (Invitrogen) was added at 30 s to cross-link CD3. At 2 min, 10 mM CaCl₂ (Sigma Aldrich) was added and the cells run until 5 min. At 5 min, 1 µg/ml ionomycin (EMD Biosciences, La Jolla, CA) was added and the sample run until 7 min.

Quantitative RT-PCR

Relative expression levels of PTPN22 were measured in each cell subpopulation by quantitative real-time PCR (qPCR). Cell lysis, cDNA synthesis, and qPCR were performed using the Power SYBR Green Cells-to-CT Kit (Ambion, Austin, TX) and a Roche Lightcycler 480 (Roche Applied Science, Indianapolis, IN). Primers were purchased from SABiosciences (Frederick, MD). PTPN22 expression levels were first normalized to the expression levels of the housekeeping gene RNA polymerase II and then calculated relative to the PTPN22 expression in the CD4+ T effector cells (Teff). For error analysis, the SD of each sample was calculated by the square root of the sum of the squares of the SDs of the triplicate Cp values of the PTPN22 and RNA polymerase II. The standard deviations of each sample were used to calculate the upper and lower ranges of the relative fold expression values.

T cell suppression assay

Tregs were isolated from splenocytes using the CD4+ CD25+ Treg isolation kit according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). The remaining CD4+ CD25− cells were CFSE labeled and 3 × 10⁴ cells were plated in a 96-well plate along with 1 × 10⁵ irradiated splenocytes (30 Gy). Tregs were titrated into the cultures at varying ratios of Teff/Treg, and 1 µg/ml soluble anti-CD3 Ab (eBioscience) was added. The cells were cultured for 72 or 96 h at 37°C with 5% CO₂. Flow cytometry was carried out to assess proliferation of Teff cells.

Intracellular cytokine staining

Draining lymph nodes were harvested from mice that had been induced with experimental autoimmune encephalomyelitis (EAE) 10 d previously. Cells were counted and seeded in a 6-well plate at 1 × 10⁶ cells/ml. Brefeldin A was added to the culture at 5 µg/ml (Sigma Aldrich). Cells were stimulated with either MOG 35–55 peptide (30 µg/ml), PMA and ionomycin (5 ng/ml and 500 ng/ml, respectively), or left unstimulated for 5 h at 37°C, 5% CO₂. Cells were then harvested and fixed with Cytofix/Cytoperm buffer (BD Biosciences) for 15 min at 4˚C. Cells were then washed in Perm/Wash solution (BD Biosciences) and stained with anti-mouse IL-17A PE and IFN-γ allophycocyanin Abs (both BioLegend).

Statistical analysis

All analyses were performed using a one-way ANOVA test with Tukey post test. Exceptions are Fig. 1E, which is detailed in the quantitative PCR method described earlier, and Fig. 7A and 7B, which used a Mann–Whitney U test. All tests were performed by Prism software (GraphPad, La Jolla, CA).

Results

Tregs are increased in the thymus of PTPN22-deficient mice

As previously reported (24), we observed that PTPN22 deficiency does not affect the numbers and proportions of the major developing T cell subsets in the thymus (data not shown). To identify Tregs and their precursors among thymic CD4+ populations from wild-type (WT), PTPN22+/− (heterozygous; Het), and the PTPN22−/− (knockout; KO) mice, thymocytes were analyzed for expression of Foxp3 and CD25 (Fig. 1A). Comparing the numbers of Tregs (CD4+Foxp3+), a significantly higher ratio of Tregs to CD4 cells was observed in both the Het and the KO mice compared with

![FIGURE 2. CD25 expression on thymic Tregs and Treg precursors. (A) Mean fluorescent intensity (MFI) of CD25 on Tregs (CD4+Foxp3+CD25+) in the thymus of WT, Het, and KO mice. (B) Representative histogram of CD25 expression on Tregs in the thymus of WT (filled plot) and KO (unfilled plot) mice (Het plot was not included for clarity). (C) MFI of CD25 on Treg precursors (CD4+Foxp3−CD25−).](http://www.jimmunol.org/)

5269
that in WT (Fig. 1B, 1C). Foxp3 and CD25 staining was used to
distinguish Tregs (CD4⁺Foxp3⁺CD25⁺) from Treg precursor pop-
ulations (CD4⁺Foxp3⁺CD25⁻) (22). An increase was observed in
the proportion of both Tregs and their precursors in the CD4
compartment of the Het and KO mice compared with that in WT
(Fig. 1B, 1C). This can be seen as a significant difference when
comparing the ratio of precursors to nonprecursors (CD4⁺Foxp3⁻
CD25⁻) (Fig. 1D). Overall, these data demonstrate increased
numbers of Tregs and their precursor population in the thymus
of PTPN22-deficient mice. Furthermore, this occurs in a dose-
dependent manner with the KO mice exhibiting higher percen-
tages of Treg populations than those of the Het.

Based on these results, we would anticipate that PTPN22 should
be expressed in thymic Tregs. Examination by quantitative real-time
PCR confirmed higher levels of expression of PTPN22 in CD4⁺
Foxp3⁺ thymocytes relative to conventional CD4 single positive
thymocytes (Fig. 1E).

In addition to increased numbers of Tregs, we observed a slight but
nonsignificant increase in the level of expression of CD25 on thymic
Tregs in Het and KO mice (Fig. 2A, 2B). This difference is not
apparent at the precursor stage (Fig. 2C) but appears after Foxp3 is
expressed. We also analyzed CD122 expression, the β-chain of the
IL-2 receptor, but found no difference in its expression by PTPN22
KO, Het, or WT (Supplemental Fig. 1).

Increased signaling in PTPN22-deficient cells
As both TCR and IL-2 receptor signals are believed to be major factors
in thymic Treg development (20–22, 25), we next addressed whether
loss of PTPN22 expression would alter TCR signaling in thymocytes.
Ca²⁺ flux is a major early consequence of TCR signaling initiated by
PLCγ1 and results in NFAT translocation into the nucleus (26). CD3
stimulation resulted in a greater increase in Ca²⁺ flux in the double
positive thymocytes of PTPN22 KO mice compared with that in WT
mice (Fig. 3A) and also in the CD4⁺CD25⁺ population of thymo-
cytes, which contains both Tregs and their precursors. CD69 ex-
pression, another indicator of strength of TCR signaling (27, 28), was
slightly higher on double positive thymocytes from KO and Het mice
than on this same population from WT mice both in ex vivo, unsti-
mulated thymocytes and those stimulated overnight with anti-CD3
and anti-CD28 (Fig. 3B, 3C).

**FIGURE 3.** Signaling differences in the thymus of PTPN22-deficient versus WT mice. (A) Calcium flux in double positive and CD4⁺CD25⁺ thymocytes
derived from WT (thin line) or KO (thick line) mice. Cells were prelabeled with anti-CD3 biotin Ab and stimulated at 30 s with streptavidin. Exogenous
calcium was added at 120 s and ionomycin at 300 s. For the experiment shown, WT cells were labeled with Cy5 and mixed with unlabeled KO cells to
stimulate under identical conditions. During the same experiment, the Cy5 labeling was reversed to ensure it did not interfere with Ca flux. This is an
example of one of four separate experiments (WT, n = 8; KO, n = 8). (B) CD69 expression on double positive thymocytes from WT (filled plot), Het
(unfilled, thin line), and KO (unfilled, thick line) mice either from unstimulated thymocytes or CD3/CD28 stimulated thymocytes (overnight stimulation).
(C) Normalized mean fluorescent intensity (MFI) of CD69 on CD3/CD28 stimulated DP thymocytes. *p < 0.05.
Overall, these data suggest there is increased signaling downstream of the TCR in developing thymocytes due to a loss of PTPN22 expression.

**Tregs are increased in the periphery of PTPN22-deficient mice**

We next sought to determine whether the increased Treg numbers were maintained in the periphery of PTPN22-deficient mice. Examination of the splenic composition revealed that there may be an increase in the proportions of CD4 T cells in the spleen (Fig. 4A), and interestingly the percentage of Tregs increased resulting in a significantly higher ratio of Tregs/CD4 cells in the PTPN22 Het and KO mice compared with that in the WT (Fig. 4B, 4C). Comparison of Tregs/effector CD4 cells (CD4*CD44*Foxp3*) revealed significantly higher ratios in the PTPN22 Het and KO compared with that in the WT mice (Fig. 4D).

**CD25 is increased on peripheral Tregs from PTPN22 KO mice**

CD25 expression was also found to be increased in peripheral Tregs from PTPN22 KO mice compared with that in peripheral Tregs from WT and Het mice (Fig. 5A, 5B). After stimulation of splenocytes with IL-2, we measured p-STAT5 levels by flow cytometry as a downstream readout of signaling through CD25/IL-2 receptor. CD4*CD25* cells from PTPN22 KO mice show higher p-STAT5 and significantly more rapid kinetics of phosphorylation of the STAT5 molecule compared with those of CD4*CD25* cells from WT mice (Fig. 5C, 5D). Overall, these data show that the increased CD25 levels on Tregs from PTPN22 KO mice observed in the thymus are maintained in the periphery. This results in a greater ability to phosphorylate STAT5, an important inducer of transcription and stabilization of Foxp3 (20).

**Increased Tregs can lead to protection from EAE in PTPN22-deficient mice**

As the percentage of Tregs is increased in the periphery of PTPN22-deficient mice, we wished to determine whether this resulted in a functional effect in vivo. In vitro Treg assays using fixed numbers of Tregs to suppress the proliferation of CD4 effector cells revealed no difference in the suppressive capability of Tregs from the three genotypes (Fig. 6), and analysis of the levels of expression of functional markers used by Tregs, such as CTLA-4, revealed no distinct differences in expression among Tregs from PTPN22 KO, Het, or WT (Supplemental Fig. 2). GITR showed a slight increase in expression on splenic Tregs from KO compared with that on splenic Tregs from WT mice (Supplemental Fig. 2). However, it is often the case that changes in the ratio of Treg/Teff have profound consequences with respect to protection from autoimmune disease (29, 30).

To investigate the role played in vivo by the increased the Treg/Teff cell ratio observed in PTPN22-deficient mice, we induced EAE using MOG peptide and pertussis toxin (Fig. 7). This par-

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**FIGURE 4.** PTPN22 deficiency results in increased peripheral Tregs. Spleens were removed from WT, Het, and KO mice and stained for CD4, CD8, Foxp3, and CD25. (A) Representative CD4 versus CD8 plots for the three genotypes of mice. (B) Representative dot plots showing CD25 and Foxp3 expression of the CD4 gate on WT, Het, and KO splenocytes. (C) Treg/CD4 ratio comparing the CD4*Foxp3* cells to CD4*Foxp3* cells in the spleen of the three groups. (D) Treg/effector CD4 ratio in the spleen of the three genotypes. Effector CD4 cells are defined as CD4*CD44*Foxp3*. *p < 0.05, **p < 0.01.
particular model was selected as it is well documented that Tregs can ameliorate EAE in vivo (29, 30). We observed a protective effect by the loss of PTPN22, as WT mice exhibited significantly higher clinical scores than the Het and KO mice. Furthermore, reduction of the number of Tregs (∼50%) using a CD25-depleting Ab caused an increase in the severity of EAE in the KO mice approaching WT levels (Fig. 7B). Analysis of Th subsets and Treg numbers in the draining lymph nodes revealed that both Th1 and Th17 numbers were lower in KO and Het mice compared with those in WT (Fig. 7C). Furthermore, the Treg/Th ratio was higher in KO mice compared with that in WT mice for both Th1 (significantly) and Th17 subsets, and the overall Treg/Teff ratio was higher both immediately ex vivo and significantly higher after MOG restimulation (Fig. 7D, 7E). These data suggest that the increased number of Tregs observed in PTPN22-deficient mice has a functional consequence in an autoimmune setting.

**Discussion**

Although still somewhat controversial, recent evidence favors a model in which the autoimmune disease-associated R620W polymorphism in the Pep/Lyp phosphatase results in a reduced level of the phosphatase (12). Thus, mice deficient in PTPN22 are excellent models in which to study the consequences of this polymorphism on the immune system. Previous work on PTPN22 KO mice focused mainly on its effects on thymic selection of conventional CD4 and CD8 T cells and on peripheral naive and effector/memory T cells. No effect on thymic deletion was observed, although positive selection was enhanced (24). Accumu-
Evidences suggest that Treg development requires affinity for self-antigens, and some mutations that reduce the strength of TCR signaling during thymic selection, such as the mutation in the SH2 domain of ZAP70 that occurs in SKG mice, result in autoimmune disease due to a reduction in the production of Tregs (31). In this study, we have examined the effect of altered levels of PTPN22 expression on Treg development, homeostasis, and function. We observed that PTPN22 deficiency enhances Treg numbers in both the thymus and periphery.

Notably, when analyzing the expression of PTPN22 in different cell types, we observed an increase in PTPN22 mRNA in CD4+Foxp3+ cells in the thymus compared with that in CD4+Foxp3- cells. This is in contrast to the periphery where we have confirmed results published by Marson et al. (32) showing higher PTPN22 in Foxp3- cells compared with that in Foxp3+ cells (Supplemental Fig. 3). They suggested Foxp3 has a negative regulatory role on expression of PTPN22. We hypothesize that this difference between the thymus and periphery may be due to stronger signaling in the thymus resulting in upregulation of PTPN22 as a feedback mechanism, which is eventually reduced by Foxp3 expression in the periphery. Overall, the expression of PTPN22 in Foxp3+ cells in the thymus suggested this gene might be playing an important role in Treg development.

Work by Lio and Hsieh (22) has suggested that Treg development is a two-step process. First, TCR signaling by self-antigens results in upregulation of CD25. Subsequent signaling through IL-2 activates STAT5 and contributes to Foxp3 transcription and the acquisition of a Treg phenotype (20, 22). As reported previously, we observed that PTPN22 deficiency results in increased Ca2+ flux among double positive thymocytes, which may be responsible for the increase in Treg precursors in PTPN22 KO and

![Figure 6](http://www.jimmunol.org/) Suppressive capability of Tregs from PTPN22-deficient mice. CD4+CD25+ Tregs were isolated from splenocytes from WT, Het, and KO mice using magnetic separation. CFSE-labeled Teff (CD4+CD25-) T cells (3 × 10⁵) were cultured in the presence of 1 × 10⁵ irradiated splenocytes and soluble anti-CD3 Ab (1 μg/ml). Tregs were titrated into the cultures at the ratios indicated. The cells were cultured for 72 h, and then proliferation was assessed by flow cytometry. This experiment is representative of three separate experiments. The 96-h time point shows similar results from two separate experiments (data not shown).

![Figure 7](http://www.jimmunol.org/) PTPN22-deficient mice are protected from EAE. (A) EAE was induced in female mice at 9 wk of age by s.c. injection of MOG 35–55 peptide in CFA followed by i.p. injection of pertussis toxin on days 1 and 2. Mice were scored each day for 28 d (WT, n = 8; Het, n = 7; KO, n = 9). This experiment was performed twice with WT and KO mice, n = 4. (B) EAE was induced in WT and KO mice on day 1 as described, and mice received an i.p. injection of anti-CD25-depleting Ab on days -1 and +4 (WT, n = 5; KO, n = 4; KO + CD25 mAb, n = 4). (C) EAE was induced as described previously, and draining lymph nodes were harvested at day 10, restimulated with MOG for 5 h, and then stained for IL-17 and IFN-γ. (D) Harvested lymph node cells were restimulated with MOG peptide and stained for IL-17, IFN-γ, and Foxp3; the Treg/Th ratio is plotted for both Th17 and Th1 cells. (E) Harvested lymph node cells were stained immediately ex vivo (unstimulated, top panel) or restimulated with MOG peptide (bottom panel) as in (D). Treg/Teff ratio is plotted (effector CD4 cells are defined as CD4+CD44hiFoxp3-). *p < 0.05, **p < 0.01, ***p < 0.001.
Het mice (11). In addition, we show Ca^{2+} flux is also increased in the CD4^{+}CD25^{+} population of thymocytes in KO compared with WT mice and may account for the larger number of Tregs. Unlike conventional CD4^{+}CD25^{+}Foxp3^{+} thymocytes, the CD4^{+}CD25^{+} Foxp3^{+} Treg precursors have the ability to upregulate Foxp3 in vitro upon treatment with IL-2 (22). Therefore, it is likely the increased numbers of CD4^{+}CD25^{+}Foxp3^{+} cells leads to increased numbers of Foxp3-expressing Tregs in the thymus.

Foxp3 regulation has been shown to be dependent on a number of transcription factors. Of particular relevance to this work is the observation that there are NFAT binding sites in the Foxp3 promoter, as well as in the CD25 promoter (33, 34). We show in this study that Ca^{2+} signaling, an important event upstream of activation of NFAT, is higher in Treg precursors in the KO mice. On the basis of the current literature, we hypothesize that this leads to increased expression of Foxp3 and CD25. Thymic Tregs in PTPN22 KO mice demonstrated increased expression of CD25 compared with that of thymic Tregs in WT mice. We did not observe an increase in another common y-chain cytokine receptor, CD122 (Supplemental Fig. 1). However, CD25 signaling can initiate STAT5 signals, which lead to transcription of the Foxp3 gene (35). On the basis of these data, we propose that increased thymocyte signaling at the double positive stage of development can lead to more Tregs that are stabilized and maintained through CD25-dependent signals both in the thymus and periphery.

Peripheral Treg numbers were also increased in PTPN22 KO mice. This increase could be due to increased thymic output of natural Tregs or alternatively a change in the induced Treg compartment. We used the Helios marker to differentiate between the two subsets and found an increase in both types (Supplemental Fig. 4). However, recent reports in the literature suggest that Helios may not be as specific for thymically developed Tregs as first thought (36). Therefore, additional experiments will need to be performed to assess the effect of PTPN22 expression on peripheral Tregs. Despite increased CD25 on peripheral Tregs in KO mice and their ability rapidly to phosphorylate STAT5 in response to IL-2 stimulation, we cannot say that homeostasis of peripheral Tregs is altered. Thymic output of Tregs is increased during PTPN22 deficiency, and this is maintained in the periphery at a ratio similar to that in WT mice. This suggests that the difference is generated in the thymus and maintained in the periphery. A possible mechanism based on these data is that after increased thymic output of Tregs, peripheral signals through CD25 signaling can maintain and stabilize Foxp3 in these cells. The data suggest this is true for KO Tregs as these have more CD25 and can phosphorylate more STAT5 in response to IL-2. However, Het Tregs and WT Tregs in the periphery look similar in terms of CD25 and p-STAT5, and therefore the major difference between these mice is that thymic production of Tregs has been increased in the Het. Of interest, studies in which the numbers of Foxp3^{+} cells in the periphery of normal donors homozygous for the susceptible 620W allele and the protective allele were compared found significantly higher percentages of Tregs in donors expressing the 620W allele (X. Castro Dopico, J. Todd, and L. Wicker, unpublished observations). This further supports the hypothesis that the susceptibility allele results in reduced levels of PTPN22.

In vitro Treg assays showed no increase in the suppressive ability of Tregs from KO mice compared with that of Tregs from WT mice. However, in such assays Treg numbers were kept constant among the genotypes tested and did not reflect the true situation in vivo where we find increased numbers of Tregs. To address the functional consequence of increased Tregs, we used an in vivo model of autoimmunity. EAE is a mouse model of multiple sclerosis induced, in this case, by injection of MOG peptide with pertussis toxin to activate MOG-specific T cells. Previous studies in this model have shown that disease can be inhibited by Tregs (29, 30). After inducing the disease in WT, Het, and KO mice, we observed that Het and KO mice developed significantly less disease than the WT mice. We have shown that increased T cell signals through loss of PTPN22 can lead to increased Tregs. EAE protection in Het and KO mice could be dependent on this higher number of Tregs as depletion of these Tregs by anti-CD25 Ab increased disease severity in the KO mouse.

Our analysis demonstrates that PTPN22 can affect Treg development and as a result alter the balance of Teff cells to Tregs in the periphery. The reduction in PTPN22 does not appear to result in higher suppressive activity by these Tregs, but the increased ratio of Tregs to effector T cells can suppress the onset of EAE. The data presented in this study show that although there are more Tregs in the Het compared with the WT mice, the CD25 levels are similar and the kinetics of p-STAT5 upregulation is similar. This could explain the result in Fig. 7A showing the Het is not as protective in the initial stages of EAE as the KO mouse, which could in part result from the less stable nature of the Treg phenotype in these mice. However, the Het does show a protective phenotype over the course of the disease, and future experiments will explore whether a loss of PTPN22 could be playing a role in Th1/Th17 differentiation and/or activity.

It is of interest that multiple sclerosis is one autoimmune disease in which the risk allele of PTPN22 is not associated with increased disease (7, 8). It has been speculated that one difference between PTPN22-associated diseases (rheumatoid arthritis, T1D, systemic lupus erythematosus, and Hashimoto’s thyroiditis) and non-associated diseases (multiple sclerosis, Crohn’s disease, and colitis) is the importance of a humoral component in the former that appears to be much less prominent in the latter (7). In a recent study, the risk allele of PTPN22 has been associated with incomplete deletion of autoreactive B cells (12). It may be speculated that autoimmune diseases that are strongly associated with the presence of autoantibodies may be less susceptible to inhibition by endogenous Tregs. In fact, there is evidence that Treg numbers are not deficient during development of T1D and may in fact be increased, although their ability to survive may be impaired, in part due to defects in the CD25R–IL-2 pathway (37–39).

These data further our understanding of PTPN22 function in cell types that have not been explored previously and suggest that if risk alleles in PTPN22 result in reduced levels of phosphatase, this does not jeopardize Treg production or survival and is likely to contribute to autoimmunity through a different mechanism, such as enhanced responsiveness by effector cells, autoreactive B cells, and dendritic cells. Future studies will examine the consequence of PTPN22 deficiency in these cells.

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