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PTPN22 Controls the Germinal Center by Influencing the Numbers and Activity of T Follicular Helper Cells

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A single nucleotide polymorphism in PTPN22 (R620W), which encodes the Lyp tyrosine phosphatase, has been linked to a number of autoimmune diseases including type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, Graves’ disease, and others (1–3). Of interest, it does not increase the frequency of Crohn’s disease or multiple sclerosis (4, 5). Because the protein is expressed in essentially all bone marrow–derived cells, such disease selectivity is likely to reflect differences in the types of immune cells contributing to each disease and how the alternative alleles of the phosphatase affect the function of the various cell types. PTPN22 encodes the lymphoid tyrosine phosphatase (LYP) in humans and PEST-enriched protein phosphatase (PEP) in mice. The functional outcome of the disease-associated allele is controversial. Originally, it was thought to be a gain-of-function mutation (6–9); however, this has been challenged in recent models with suggestions that the mutation causes a loss of function (10, 11) or an alteration of substrate specificity (12).

To learn about the effect of PTPN22 on immune cells, several laboratories have produced knockout (KO) mice (13, 14). When on the B6 background, mice deficient in PEP show no overt autoimmune disease, although they do exhibit splenomegaly and increased effector/memory T cells that accumulate over time. This phenotype is attributable to the fact that PEP targets include the Src-family kinases including signaling molecules proximal to the TCR, including Lck, Fyn, and ZAP70 (15, 16). In the absence of PEP, T cell signaling is increased (13), mice exhibit greater numbers of germinal centers (GCs), and they have higher levels of IgG in their sera compared with wild type (WT) mice. Despite this observation, B cell signaling was reported to be similar between WT and KO mice, suggesting this is an indirect effect attributable to the enhanced activity of T cells (13).

PTPN22 also influences regulatory T cell (Treg) number and function, which is important in the context of autoimmunity. We have shown previously that PTPN22 deficiency increases thymic development of Tregs, leading to an increase in the numbers of peripheral Tregs (17). This increase has been confirmed by other groups in various PTPN22 models (9, 14). It has also been reported that Treg suppressive function is enhanced through an LFA-1–mediated mechanism in PTPN22 KO mice (14).

PTPN22 is expressed in B cells, although at a lower level than in T cells (18). The majority of studies on the effect of PTPN22 in B cells have been performed by comparing human samples that carry the R620W variant and the common allele. These studies have suggested the R620W allele impairs BCR signaling, leading to expansion of transitional and anergic B cells that exhibit reduced apoptosis upon BCR engagement (19, 20). Another study has also reported that the risk variant leads to escape of autoimmune B cells through a defect in central and peripheral tolerance (21). Mouse studies have shown that PTPN22 KO mice can develop a lupus-like phenotype when bred to mice containing a mutation in CD45, resulting in increased B cell activation (11).

Recently, two groups have introduced the disease-associated allele (PEP<sup>R619W</sup> analogous to the human LYP<sup>R620W</sup>) and found that these knock-in mice exhibit a phenotype similar to the KO mouse with increased GCs and increased serum IgG (10, 12). One of these studies suggested that on a mixed genetic background (129/Sv background several generations to B6), B cell tolerance was impaired in the PEP<sup>R619W</sup> mouse (12). Finally, in a recent report, PTPN22 expression in the NOD mouse was knocked down by RNA interference, resulting in enhanced B cell upregulation of CD25 and CD69 in response to anti-IgM and anti-CD40 ligation. In addition, apoptosis was increased, suggesting the KO has the
opposite effect of the R620W allele on human B cells and consistent with the hypothesis that R620W increases the phosphatase activity of PEP/LYP (9). However, because of the nature of the autoimmune prone backgrounds thus far reported, the B cell phenotypes described would be difficult to attribute solely to a mutation in PTPN22. Working with a nonautoimmune prone background such as the B6 PTPN22 KO mouse allows us to identify PTPN22-dependent phenotypes in the absence of genetic interactions with other pro-autoimmune alleles that can alter immune phenotype.

Efficient Ab generation by B cells relies on the formation of a GC in which optimal interaction between T and B cells occurs (22). T follicular helper cells (Tfh) are a subset of CD4⁺ T cells that express CXCR5 and migrate to the GC via a CXCL13 chemokine gradient to provide help to B cells (23–26). Help in this case can be through production of effector cytokines IL-21 (27, 28) and IL-4 (29, 30), as well as engagement of CD40 on B cells through CD40L expression, promoting activation of B cells and class switching, as well as formation of plasma cells (31, 32). Interaction with dendritic cells in the initial stages of activation of CD4⁺ T cells is thought to determine the fate and differentiation of Th0 cells into Tfh, controlled by the transcription factor Bcl-6 (33–35). Further interaction between B cells stabilizes this phenotype (22). Strength of signal during these early stages may favor Tfh differentiation, a point of particular relevance in the case of PTPN22 KO mice (29). Regulation of the GC is crucial to maintaining peripheral tolerance. A recently described population of lymphically derived Tregs that express CXCR5 and are also localized to the GC, known as follicular regulatory T cells (Tfr), can control the GC reaction (36, 37). It has been suggested that the balance of Tfr: Tfh is important in controlling the magnitude of the humoral immunity (38).

In this study, we have used PTPN22 KO mice to investigate the mechanisms by which PTPN22 may alter formation and regulation of the GC. Overall understanding of how this critical disease susceptibility gene functions in humoral immunity will allow us to identify targets in a number of autoimmune conditions that may be beneficial for therapy. We have found that loss of PTPN22 increases GC activity predominantly by increasing proliferation, survival, and cytokine secretion by Tfh. Through adoptive transfer models, we show that the T cell genotype is more important than the B cell genotype in terms of the contribution of PTPN22. We also find that the ratio of Tfr/Tfh does not increase in PTPN22 KO mice, which we hypothesize may be because of an observed increase in IL-21 production in the GC. We propose the Tfr are unable to control the expanded number of Tfh present in PTPN22 KO mice, resulting in enhanced Ab production.

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. PTPN22 KO mice were obtained from Dr. Andrew Chan (Genentech, San Francisco, CA) and have previously been described (13). PTPN22 wild-type mice were obtained by interbreeding PTPN22+/− mice with C57BL/6 (The Jackson Laboratory, Bar Harbor, ME). Foxp3 GFP and OT-II Thy 1.1+ mice were provided by Dr. Charles Surh (The Scripps Research Institute [TSRI]) and were bred to PTPN22−/− mice. Nur-77 GFP knock-in mice were provided by Dr. Kristin Hogquist (University of Minnesota) and bred to PTPN22−/− mice (39). Kbxn mice were produced through breeding of KRN mice (provided by Dr. Kerri Mowen, TSRI) with B6.H2g7 mice (The Jackson Laboratory). PTPN22−/− mice were bred to KRN and/or B6.H2g7 mice to produce PTPN22−/−/Kbxn mice. 8- to 12-wk-old mice were immunized s.c. in the flank with 100 μg 4-hydroxy-3-nitrophenylacetyl conjugated keyhole limpet hemocyanin (NP-KLH) (Biosearch Technologies, Novato, CA) or OVA (Invivogen, San Diego, CA) in CFA (Difco, Detroit, MI). Secondary immunizations were carried out by injecting the same dose of Ag s.c. without CFA, 14 d after primary immunization. The draining lymph node (dLN) refers to the inguinal lymph node on the side of the mouse being injected.

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 Bioscience, Franklin Lakes, NJ) and analysis using FlowJo (Tree Star).

Abs (all Biolegend, San Diego, CA, unless otherwise stated) used were anti-mouse CD4 PerCP Cy5.5, CD8 Pacific Blue/allophycocyanin Cy7, PD-1 FITC/PE, CXCR5-biotin (BD Biosciences), CD44 Pacific Blue, GL-7 FITC, FAS PE, CD138 allophycocyanin, CD19 PerCP Cy5.5, CD23 PE, CD21 PerCP Cy5.5, streptavidin allophycocyanin. For intracellular staining of markers, an intracellular staining kit (Fix/Perm; eBioscience, San Diego, CA) was used together with anti-mouse Foxp3 PE (eBioscience) and Ki-67 allophycocyanin (Biolegend). Intracellular cytokine staining was carried out using Cytofix/Cytoperm buffer (BD and IL-2 PE; 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 Bioscience) or MoFlo XDP (Beckman Coulter, Indianola, IN) by TSRI flow cytometry core facility.

Calcium flux

Splenocytes were rested in complete RPMI 1640 (cRPMI) for 20 min at 37°C before staining. Splenocytes 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 CD19 FITC, CD23 PE, and CD21 PerCP Cy5.5 (all from Biolegend), resuspended in HBSS (Life Technologies), and kept on ice. Cells were then warmed to 37°C before running the sample and ran for 30 s to establish a baseline; 10 μg/ml anti-IgM Fab2 (Jackson ImmunoResearch Laboratories, West Grove, PA) was added at 30 s to stimulate B cells. At 2 min, 10 nM CaCl₂ (Sigma Aldrich, St. Louis, MO) was added and the cells were 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.

Lymphocyte purification

FACS sorting was carried out as described in the earlier method. Magnetic separation of CD4⁺ T cells was carried out using a CD4⁺ depletin kit according to manufacturer’s instructions (BD). B cells were isolated from splenocytes using a B220⁺ selection kit according to manufacturer’s instructions (Miltenyi Biotec, Auburn, CA).

ELISA

Serum was collected from mice at the stated time points. Maxisorp plates (Nunc, Rochester, NY) were coated with 5 μg/ml OVA (Invivogen) or NP-BSA (Biosearch Technologies; NP4 for high-affinity Abs or NP32 for broad-affinity Abs) overnight at 4°C. Plates were blocked in 5% BSA (Sigma-Aldrich) for an hour at 37°C. Plates were washed three times with wash buffer (HBSS with 0.1% Tween 20, Sigma-Aldrich). Sera were diluted accordingly after optimization for each experiment in reagent buffer (HBSS containing 1% BSA, 0.1% Tween 20) and incubated on the plate in triplicate for 1 h at 37°C. Plates were washed three times. Anti-mouse IgG alkaline phosphatase or anti-mouse IgG1 HRP were then diluted and added to the wells for a further hour at 37°C (both from Jackson ImmunoResearch Laboratories). Plates were washed and then incubated with either pNP alkaline phosphatase substrate or TMB HRP substrate (both from Sigma-Aldrich). Plates were read using a Versamax plate reader (Molecular Devices, Sunnyvale, CA) at 405 or 450 nm, respectively.

Quantitative real-time PCR

NP-KLH-immunized dLNs were collected from Foxp3-GFP PTPN22 WT and KO mice 7 d postimmunization. The cells were stained for CXCR5, CD44, CD4, and PD-1, and FACS sorted for Tfh (CD4⁺ CD44⁺CXCR5⁺ PD-1⁻ Foxp3⁻) and Tfr (CD4⁺ CD44⁺CXCR5⁺ PD-1⁺ Foxp3⁺). mRNA was then extracted by TRizol (Life Technologies, Carlsbad, CA), and cDNA was produced using the high-capacity cDNA RT kit (Applied Biosystems, Carlsbad, CA) according to manufacturer’s instructions. Quantitative real-time PCR (Q-PCR), using a fixed amount of cDNA produced from comparable numbers of cells from WT and KO mice, was performed to...
measure levels of IL-21, IL-4, IL-10, Bcl2, Mcl-1, Bim L, Bim EL, and Bax (sequences available on request) using a 7900HT real-time PCR system (Applied Biosystems). Threshold cycle values were normalized to β2M using the 2^−ΔΔc threshold cycle method. KO values were normalized to WT.

**In vitro B cell help assay**

PTPN22 KO and WT mice were immunized with NP-KLH as described earlier. Eight days later, dLNs were extracted and CD4 T cells were purified as described earlier. Unimmunized PTPN22 WT and KO mouse spleens were also collected at this time point, and B cells were purified as described earlier. CD4 T cells (5 × 10^5/well) and B cells (5 × 10^5/well) were cocultured in the combinations stated in the Results with 20 ng/ml IL-2, 2-ME, and NP-KLH (Biosearch Technologies) for a further 7 d at 37°C. Supernatant was collected and analyzed for NP-specific IgG by ELISA as described earlier.

**Confocal microscopy**

Lymph nodes were collected at the stated time points postimmunization, immersion-fixed in 1% v/v paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). LNs were then transferred to 15% sucrose (Sigma-Aldrich) until the organs sank and then frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA). LN sections were cut at 10 μM using a Leica CM1850 cryostat (Leica Microsystems, Buffalo Grove, IL). Sections were acetone (Sigma Aldrich) treated for 10 min at room temperature and then washed in PBS. Abs used to stain for markers are as follows: anti-mouse CD4 (eBioscience), peanut agglutinin–biotin (Biosearch Technologies), anti-rat AF647, streptavidin AF555 (both Life Technologies, Carlsbad, CA). Images were acquired on a Leica 780 confocal microscope (Leica Microsystems, Buffalo Grove, IL) and analyzed with Imaris (Bitplane, South Windsor, CT) and Image Pro Plus software (Media Cybernetics, Rockville, MD).

**FIGURE 1.** Numbers and intracellular signaling of B cells in PTPN22 KO mice. Spleens were taken from WT and KO mice, and stained for follicular (CD19+ CD21+ CD23+) (A) and MZ B cells (CD19+ CD21hi CD23lo) (B), and absolute numbers in the spleen are shown (graphs show mean ± SEM; each dot represents one mouse). Splenocytes were labeled with Indo-1, and calcium flux was measured for both follicular and MZ B cells in response to anti-IgM at 30 s and Ionomycin at 3 min (C) (thick line = WT; thin line = KO). Nur-77 GFP PTPN22 WT (filled gray) and KO (unfilled black line) spleens were analyzed for GFP expression at ex vivo or after 3-h anti-IgM stimulation. GFP expression of follicular B cells is shown in (D) and MZ in (E). (C–E) Representative of two independent experiments. *p < 0.05.

**Scoring of KBxN arthritis**

KBxN mice were weaned at 3 wk of age, and paw thickness (front and hind) was measured by calipers. Clinical scoring was based on previously described scoring criteria (40). Serum-transferred arthritis was induced in PTPN22 KO and WT mice through i.p. injection of 150 μl serum collected from 6- to 10-wk-old KBxN mice.

**Statistics**

Graphs were assembled and analyzed using Prism 5 software (GraphPad, San Diego, CA). For multiple-group analyses, one-way ANOVA with Tukey’s posttest was carried out. Student t test was used for comparison of two group data sets.

**Results**

**PTPN22 KO mice have increased follicular B cells**

Following the observation that PTPN22 KO mice had increased numbers of GCs, we analyzed spleens of unimmunized mice for the distribution of cells among the various B cell subsets by flow cytometry (Fig. 1). PTPN22 KO mice have significantly more follicular B cells compared with WT mice; however, marginal zone (MZ) B cell numbers were similar in both types of mice (Fig. 1A, 1B). Calcium flux in response to anti-IgM stimulation was also similar between PTPN22 WT and KO B cells of both follicular and MZ subsets (Fig. 1C). We used Nur-77 GFP mice as a reporter of strength of BCR signaling to measure the response to anti-IgM stimulation (41). Follicular (Fig. 1D) and MZ (Fig. 1E) subsets from PTPN22 WT and KO mice show no difference in resting GFP levels or in response to IgM stimulation. Overall, these data suggest an
accumulation of follicular B cells occurs in PTPN22 KO mice, but intrinsic signaling differences were not detectable by calcium flux or activation of Nur-77.

**PTPN22 KO mice demonstrate increased responses to T-dependent Ag**

To assess the influence of PTPN22 on GC activity, we immunized mice with NP-KLH in CFA (Fig. 2) and analyzed cells from the dLNs at various time points postimmunization. In agreement with previous reports (13), we observed larger and more numerous GCs in KO mice compared with WT mice (Fig. 2A). We also observed that both the percentage and absolute number of T\(_{FH}\) in the dLNs was increased in KO mice. This peaked 7 d postimmunization and reached statistical significance (Fig. 2B, 2C). In a similar manner, numbers of GC B cells were significantly increased in dLNs of PTPN22 KO mice compared with WT mice (Fig. 2D). NP-specific Ab levels in the sera of these mice 14 d postimmunization was quantified by ELISA (Fig. 2E). There was a significantly higher level of broad-affinity anti-NP IgG in KO mice compared with WT, as well as slightly higher, yet not statistically significant, levels of high-affinity anti-NP IgG.

We next assessed the recall response to Ag. Mice were immunized with NP-KLH in CFA and then rechallenged with NP-KLH. Analysis of the dLNs was carried out 7 d after rechallenge. Similar to the primary response, PTPN22 KO mice show significantly higher numbers of T\(_{FH}\) and GC B cells (Fig. 3A, 3B). Numbers of plasma cells were also significantly increased in KO dLNs compared with WT (Fig. 3C). Surprisingly, the numbers of plasma cells in the WT dLNs were low. We hypothesize that the majority of these may have migrated to the bone marrow by this time. Anti-NP-specific IgG, of both broad and high affinity, were significantly increased in the sera of PTPN22 KO mice compared with WT mice (Fig. 3D). The significant increase in the KO of high-affinity Abs in the secondary response compared with the primary response suggests the effect of the KO increases over time. Overall, these data are consistent with increased GC activity in PTPN22 KO mice resulting in higher levels of Ab production.

**T\(_{FR}\) are not upregulated in PTPN22 KO mice**

We, as well as others, have previously shown that PTPN22-deficient mice have increased numbers of Tregs (9, 14, 17). This may help compensate for the increased activity of conventional CD4 T cells (Tconvs). Recently, a subset of thymically derived Tregs has been described that specifically regulates the GCs (36, 37). These T\(_{FR}\) express CXCR5, which allows them to migrate to the follicle in the same manner as T\(_{FH}\). In an attempt to reconcile the fact that PTPN22 KO mice have increased Tregs but also increased GC activity, we assessed the ratio of T\(_{FR}\) and T\(_{FH}\) in dLNs 7 d postimmunization (Fig. 4). As previously shown, the Treg/Tconv ratio is increased in PTPN22 KO mice compared with WT mice; however,
the Tfr/Tfh ratio was found to be similar in both WT and PTPN22 KO mice (Fig. 4A). The spleen in these same mice showed a reduced Tfr/Tfh ratio in KO mice compared with WT (Supplemental Fig. 1). Analyzing both total cell numbers and cell proliferation (by Ki-67 staining), we found that Tconv were similar in number in both WT and KO mice, whereas the Treg numbers were significantly higher in KO mice (Fig. 4B). Numbers of dividing (Ki-67+) cells were similar between WT and KO mice in both the CD4+ Tconv population and the Treg population (Fig. 4C).

When analyzing the numbers of CD4+ cells within the follicular subset (CXCR5+ PD-1+), we found Tfh numbers to be significantly higher, and more of them are in the cell cycle (higher percentage of
Ki-67+ cells) in the KO mouse as compared with the WT (Fig. 4D, 4E). In contrast, the absolute number and the number of Ki-67+ T<sub>FR</sub> was the same in WT and KO mice (Fig. 4D, 4E).

In addition to a proliferative advantage by PTPN22 KO T<sub>FH</sub>, mRNA levels of Bcl2 were higher in this population compared with WT (Fig. 4F), suggesting a survival advantage. Bcl2 mRNA in T<sub>FR</sub> was similar in both genotypes. Analysis of further apoptosis-associated markers was carried out by Q-PCR and is shown in Supplemental Fig. 2. Among T<sub>FH</sub>, the prosurvival molecule Mcl-1 and the proapoptotic Bim L and Bax were unchanged between WT and KO. The proapoptotic Bim EL splice variant was upregulated slightly in KO T<sub>FR</sub>, although not to the levels observed with Bcl-2. KO T<sub>FR</sub> showed a slight increase in Mcl-1 transcript, but the other apoptotic markers were similarly expressed in WT and KO. Overall, because of the large increase in Bcl-2 and relatively small changes to other molecules, these data suggest that PTPN22 KO T<sub>FR</sub> are more protected from apoptosis than WT, which could contribute to the increased numbers observed in the LN.

PTPN22 KO T<sub>FH</sub> produce more IL-21 and IL-4 than WT T<sub>FH</sub>

The disparity between Tregs and T<sub>FR</sub> in the KO mouse may arise from differences in their cytokine environments. T<sub>FH</sub> can produce high levels of IL-21 that destabilize Foxp3 and promote Bcl-6 transcription, the master regulator of T<sub>FH</sub> differentiation (33–35). Analysis of total mRNA from the dLNs showed a significantly higher level of IL-21 transcript in KO mice compared with WT (Fig. 5A). Indeed, IL-21+ T<sub>FH</sub> were present in greater numbers (Fig. 5B), and we observed higher IL-21 transcript levels in a sorted population of PTPN22 KO T<sub>FH</sub> (Fig. 5C) compared with WT T<sub>FH</sub>. KO T<sub>FH</sub> also expressed more IL-4 mRNA (Fig. 5D) than WT, a cytokine that provides B cell help. In contrast, IL-10 was similarly expressed in WT and KO T<sub>FR</sub>. To date, this is the only cytokine to be associated with their function (37) (Fig. 5E).

PTPN22 KO CD4 T cells provide increased help to B cells

To further dissect the cellular basis for how PTPN22 affects the GCs, we used a TCR transgenic adoptive transfer model (Fig. 6). Thy 1.1–labeled PTPN22 WT or KO OT-II CD4 T cells were purified and transferred into either WT or KO hosts. Two days later, these mice were immunized with OVA in CFA, and the dLNs and sera were collected at various time points. The numbers of GC B cells in the dLN were measured by flow cytometry at 11 d post-immunization (Fig. 6A). In the presence of PTPN22 KO OT-II cells, GC B cell numbers were significantly higher than in the presence of WT OT-II cells, regardless of host genotype. In a similar manner, plasma cell numbers were increased in the presence of PTPN22 KO OT-II cells. This difference is significant in the WT host but not in the KO host (Fig. 6B). This was also reflected in the level of anti-OVA IgG1 in the sera at day 14 (Fig. 6C). In addition, there was a small increase in the percentage of IL-21+ OT-II T cells in the mice that received the PTPN22 KO OT-II cells as compared with WT OT-II T cells (Fig. 6D). This was significant in the case of the KO host but did not reach statistical significance in the WT host. Overall, these data suggest that PTPN22 deficiency in CD4 T cells is sufficient to provide improved help to B cells.

Increased GC activity in PTPN22 KO mice is influenced by T cells rather than B cells

The OT-II transfer model allowed us to examine how the loss of PTPN22 in the T cell would affect the GC; however, we also wished to measure a polyclonal response by a conventional repertoire with a normal ratio of T<sub>FR</sub>/T<sub>FH</sub>. To this end, we used sublethally irradiated PTPN22 WT hosts and transferred CD4<sup>+</sup> T cells and B cells purified from either PTPN22 WT or KO mice (Fig. 7). Two days later, mice were immunized with NP-KLH, and the B cell response was analyzed 14 d later.

GC B cell numbers and NP-specific Abs titers (Fig. 7A, 7B) were dependent on T cell rather than B cell genotype because the numbers of T<sub>FR</sub> in mice that received PTPN22 KO CD4 T cells were increased compared with those that received WT CD4 T cells (Fig. 7C). Looking specifically at anti-NP titers, there are significant increases in broad-affinity (NP32) Ab if the mice received KO CD4 T cells compared with WT T cells. Similarly, there are trends toward higher levels of high-affinity Ab (NP4) in mice that received KO CD4 T cells compared with WT, although not to significant levels. This may reflect the fact that the assay is measuring the primary response. As previously described, high-affinity Ab levels were significantly increased in secondary responses in KO mice (Fig. 3), whereas there was no statistical significance in the increase observed in the primary response of KO mice compared with WT (Fig. 2). Overall, the data in Fig. 7 show GC activity was increased in mice with PTPN22 KO CD4 T cells.

In addition to this in vivo model, we used an in vitro approach to determine the influence of PTPN22 on T and B cells by adapting a model described by Yusuf et al. (30) (Fig. 7D). WT and KO mice were immunized with NP-KLH in CFA, and after 7 d the LNs were removed and CD4 T cells isolated. The purified cells were

**FIGURE 5.** Cytokine production in PTPN22 KO mice. Foxp3-GFP PTPN22 WT and KO mice were immunized with NP-KLH in CFA for 7 d. Total mRNA from the dLN was extracted, and the levels of IL-21 transcript were measured by Q-PCR (A). The number of IL-21+ T<sub>FH</sub> in the dLN 7 d post-immunization was measured by intracellular cytokine staining (B). Cells from the dLN were FACSorted into T<sub>FH</sub> (CD4<sup>+</sup> CXCR5<sup>+</sup> PD-1<sup>-</sup> Foxp3<sup>+</sup>) and T<sub>FR</sub> (CD4<sup>+</sup> CXCR5<sup>-</sup> PD-1<sup>+</sup> Foxp3<sup>-</sup>) populations. mRNA was purified and cytokines were measured by Q-PCR. T<sub>FH</sub> mRNA levels of IL-21 (C) and IL-4 (D) were measured, as well as IL-10 (E) mRNA levels in T<sub>FR</sub>. All Q-PCR data shown are normalized to WT levels. (A) A combination of n = 3 mice per group. (B) Representative of three independent experiments (each data point represents one mouse). (C–E) Result of three independent sorts combined with a total of four LNs pooled per sort, per genotype (graphs show mean ± SEM). *p < 0.05.
cocultured with B cells from either WT or KO mice for a further 8 d, and anti-NP IgG in the supernatant was measured. The presence of KO CD4 T cells significantly increased anti-NP IgG production regardless of the genotype of the B cells. This further supports the conclusion that T cell help in PTPN22 KO mice is increased compared with WT mice and is responsible for increased Ig.

**FIGURE 6.** PTPN22 KO CD4 T cells can provide improved help to B cells. Thy 1.1+ OT-II+ PTPN22 WT and KO CD4+ T cells were isolated by MACS and (2 × 10^6) injected into WT or KO hosts. Mice were immunized with OVA in CFA s.c., and the dLN and sera were analyzed 14 d later. (A) Number of GC B cells (CD19+ FAS+ GL-7+). (B) Number of plasma cells (CD19hi CD138+). (C) Anti-OVA IgG1 in the sera measured by ELISA. (D) OT-II IL-21+ cells in the dLN 7 d postimmunization as detected by intracellular cytokine staining. Graphs show mean; error bars = SEM, and each data point in (A)–(C) represents one mouse. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 7.** T cell PTPN22 genotype is dominant in controlling B cell responses. WT and KO CD4+ T cells (5 × 10^6) and B cells (1 × 10^7) were isolated by MACS and injected into irradiated WT host mice. Mice were then immunized with NP-KLH in CFA s.c., and the sera and spleens were analyzed 14 d later. x-Axis labels denote the PTPN22 genotype of the cells. (A) GC B cell (CD19+ FAS+ GL-7+) numbers shown. (B) Sera anti-NP IgG of broad affinity (NP32) and high affinity (NP4; n = 5 mice/group). TFH numbers were counted in the spleens of mice (C). Graphs show mean ± SEM; data points in A and C represent one mouse. (D) PTPN22 KO and WT mice were immunized with NP-KLH s.c. Seven days later, dLNs were removed and CD4 T cells were isolated and cocultured with B cells isolated from either WT or KO spleens (unimmunized) for a further 8 d in vitro. NP-specific IgG was measured by ELISA (n = 6 mice/group). *p < 0.05, **p < 0.01, ***p < 0.001.
KBxN arthritis severity is increased in PTPN22-deficient mice

Mice expressing the KRN TCR and carrying the Aβ7 MHC molecule (KBxN) experience development of severe arthritis. This arthritis is characterized by autoantibodies against GPI (42). PTPN22 KO, Het, and WT KBxN mice were monitored for arthritis by paw thickness measurements (Fig. 8A) and clinical scoring (Fig. 8B). PTPN22 deficiency significantly increased the severity of disease, and PTPN22 KO KBxN mice either died early (around 32 d of age) or were euthanized because of complications as a result of severe arthritis (e.g., weight loss). Serum from KBxN mice can transfer disease to B6 mice (42). Serum from PTPN22 WT KBxN mice was transferred into PTPN22 WT and KO B6 mice to assess the role of PTPN22 deficiency in events downstream of Ab production (Fig. 8C). There was no difference in either the incidence or the severity of disease between these hosts, indicating that PTPN22 was not influencing events downstream of autoantibody production.

At 28 d of age, spleens were collected from PTPN22 KO and WT KBxN mice and analyzed for numbers of TFH, TFR, and GC B cells. TFH numbers were significantly higher in PTPN22 KO mice compared with WT (Fig. 8D), and the TFR/TFH ratio was also decreased (Fig. 8E). GC B cell frequency was significantly increased in the spleens of PTPN22 KO KBxN mice compared with WT (Fig. 8F). Taken together, these results indicate that PTPN22 deficiency in the induction phase of KBxN arthritis, but not the later effector stage, can increase disease severity. This was associated with a greater expansion of TFH at a young age in PTPN22 KO KBxN mice compared with WT KBxN mice.

Discussion

Although autoimmunity is not associated with deficiency of PTPN22, there is a consistent increase in GC activity and serum levels of Ig in both PTPN22 KO and mice expressing the susceptibility allele. This study was undertaken to identify the cellular and molecular events by which PTPN22 contributes to GC activity and Ab production. PTPN22 KO mice show increased GC formation and accumulation of follicular B cells; however, there appears to be little difference in the intrinsic signaling properties of B cells in the KO mouse compared with WT (Fig. 1) (13). Through investigation of key GC cell types, we found that the increased GC activity and Ab levels in PTPN22 KO mouse can largely be attributed to the T cell compartment. Postimmunization, we observed significantly greater expansion of TFH in KO compared with WT mice. There were more Ki-67+ TFH and they expressed higher transcript levels of the prosurvival molecule, Bcl-2, in the KO than in the WT dLN. Through adoptive transfer experiments of CD4 T cells, we demonstrated that KO CD4 T cells, which include the TFH population, could provide improved help to B cells regardless of the B cell genotype. One possible reason for this improved help, aside from a larger expansion of this population, is that KO TFH produce higher levels of IL-21 compared with WT TFH. This cytokine is important in two ways: first, it provides help to GC B cells allowing class switching and differentiation into plasma cells (27, 28); and second, in the environment of the GC, we hypothesize that this may have an inhibitory/destabilizing effect on TFR. TFR are a recently described subset of thymically derived Tregs (36, 37). These cells express CXCR5 and PD-1, allowing them to migrate to the GC, and are important in controlling GC reactions. PTPN22 KO mice have increased numbers of Tregs in the periphery compared with WT mice; however, despite their increased numbers, we have shown that GC reactions are amplified, suggesting lack of control specifically in the GC. Analysis of the TFR/TFH ratio in the dLN after immunization showed that PTPN22 KO and WT mice have similar (Fig. 2), or possibly lower (Supplementary Fig. 1), ratios of TFR to TFH. This is in contrast with the conventional Treg compartment, which is expanded in the KO mouse. There are significantly more Ki-67+ KO TFH than their WT counterparts.
counterparts; however, this is not the case in the T\textsubscript{FR} population, and as such the compensatory increase in Tregs that occurs elsewhere in the KO mouse is absent in the GC. We hypothesize that this is due to the higher level of IL-21 in the lymph node, produced by overactive and greater numbers of T\textsubscript{FH} in the PTPN22 KO. IL-21 is inhibitory to Treg function and stability because it signals downstream through STAT3 rather than STAT5, resulting in Foxp3 instability (43–45). Because of the localized production of IL-21 by TFH in the follicle, this does not affect the stability of the conventional Treg population that is located in the T cell zone of the LN. Overall, T\textsubscript{FH} in the GCs of PTPN22 KO mice overwhelm the T\textsubscript{FR} and the balance is shifted in favor of B cell activation.

This unique alteration in the balance of Treg/effectort T cells in different locations could possibly explain the association of certain diseases with PTPN22. A predominantly Th1/Th17-driven disease such as multiple sclerosis has no association with PTPN22, and in a mouse model of experimental autoimmune encephalomyelitis, the increased Treg/CD4 ratio was sufficient to protect the KO mice from disease (4, 17). However, in an Ab-mediated disease, loss of PTPN22 may not prevent disease despite the overall increase in Treg numbers because the specific Treg subset that controls the GC, the T\textsubscript{FR} population, does not expand in the same manner.

The effect of PTPN22 on the GC has a clear effect on an autoimmune prone background. Disease in the KBxN model has been associated with many of the changes we find associated with PTPN22 deficiency. Loss of Tregs in KBxN scurfy mice leads to an increase in T\textsubscript{FH} and disease (46). Deletion of the IL-21R on the KBxN background resulted in mice refractory to disease (47). We show that arthritis in the KBxN model was increased with PTPN22 deficiency and associated with increased frequency of T\textsubscript{FH} and a lower T\textsubscript{FRI}/T\textsubscript{FH} ratio. PTPN22 is important in the induction phase of this model, however, not the later stages downstream of Ab production as highlighted by our serum transfer data (Fig. 8C), which confirms a recent report by Wang et al. (48).

The cell transfer studies presented in this article provide the first clear evidence that a deficiency in PTPN22 in T cells can provide increased help to B cells regardless of B cell genotype. As previously suggested by Hasegawa et al. (13), we find that the increased numbers of GCs observed B6 PTPN22 KO mouse models is directly related to T cell hyperactivity rather than aberrations in B cell function. T\textsubscript{FH} differentiation is thought to be a multistep process that involves first interaction with Ag-presenting dendritic cells to induce Bcl-6 expression and then further interaction with B cells in the follicle to stabilize this phenotype (22). Because the B cell genotype had no effect in the cell transfer models, we hypothesize that the difference between WT and KO T\textsubscript{FH} is initiated at the time of DC priming of the T cell. It has been reported that strength of signal between the T cell and DC can influence the differentiation of a T cell to the T\textsubscript{FH} lineage, with stronger interactions favoring T\textsubscript{FH} commitment and IL-21 production (29).

It is known that in the absence of PTPN22 or in PTPN22 R620W knock-in mice, proximal TCR signaling is increased, particularly in the CD44\textsuperscript{hi} compartment (10, 12–14). We show PTPN22 KO T cells differentiate more readily into T\textsubscript{FH} compared with WT T cells and produce more IL-21 (Figs. 2, 3, 5). In addition, monoclonal PTPN22 KO OT-II CD4\textsuperscript{+} T cells produce more IL-21 than WT OT-II cells, despite having equal affinity TCRs (Fig. 6). The strength of signal in the case of PTPN22 KO OT-II cells would be higher than WT OT-II cells. Once committed, further TCR signals received by the KO T\textsubscript{FH} will result in greater proliferation and cytokine production, much in the same way that has been reported for effector/memory T cells in this KO mouse (13, 14). Combined with a lack of T\textsubscript{FR} expansion to match the increased activity of the T\textsubscript{FH} in the KO mouse, this leads to greater GC activity.

The exact mechanism by which the R620W allele of LYP enhances autoimmune diseases is not certain. Reports have argued a gain of function (6–9), as well as a loss of function, in lymphocytes (11). Recently, two mouse studies using the PEP\textsuperscript{R620W} knock-in mice have reported conflicting hypotheses as to the impact of this allele (10, 12), the most recent of which demonstrates that the former report, suggesting PEP\textsuperscript{R620W}, is susceptible to increased protein degradation, was possibly an artifact of the Ab used to detect this protein. Nevertheless, both studies show a phenotype closely resembling the PTPN22 KO mouse. Our study suggests that loss of PTPN22 results in increased GC activity, a phenotype that would expect to lead to an increase in disease in an Ab-mediated autoimmune condition driven by other genetic loci, arguing that the R620W allele associated with disease would result in a loss of function. However, as hypothesized by Dai et al. (12), the introduction of the R620W allele could lead to an alteration of substrate specificity for the protein and as such lead to a loss of function with some substrates and a gain of function with others. The “loss of function” forced through KO of PTPN22 in the mice in this study would suggest that this effect is sufficient to trigger increased GC activity. However, in the presence of the R620W allele, this phenotype may be more subtle and the outcome could be influenced by other disease susceptibility loci, which may also result in the loss of B cell tolerance through endogenous effects (10, 12).

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References


