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PTPN22 silencing in the NOD Model Indicates the Type 1 Diabetes–Associated Allele Is Not a Loss-of-Function Variant

Peilin Zheng1 and Stephan Kissler1,2

PTPN22 encodes the lymphoid tyrosine phosphatase (LYP) and is the second strongest non-HLA genetic risk factor for type 1 diabetes. The PTPN22 susceptibility allele generates an LYP variant with an arginine-to-tryptophan substitution at position 620 (R620W) that has been reported by several studies to impart a gain of function. However, a recent report investigating both human cells and a knockin mouse model containing the R620W homolog suggested that this variation causes faster protein degradation. Whether LYP R620W is a gain- or loss-of-function variant, therefore, remains controversial. To address this issue, we generated transgenic NOD mice (nonobese diabetic) in which Ptpn22 can be inducibly silenced by RNA interference. We found that Ptpn22 silencing in the NOD model replicated many of the phenotypes observed in C57BL/6 Ptpn22 knockout mice, including an increase in regulatory T cells. Notably, loss of Ptpn22 led to phenotypic changes in B cells opposite to those reported for the human susceptibility allele. Furthermore, Ptpn22 knockdown did not increase the risk of autoimmune diabetes but, rather, conferred protection from disease. Overall, to our knowledge, this is the first functional study of Ptpn22 within a model of type 1 diabetes, and the data do not support a loss of function for the PTPN22 disease variant. Diabetes 62:896–904, 2013

PTPN22 encodes the lymphoid tyrosine phosphatase (LYP) (1) that has been shown to modulate the activation of both T and B cells (2,3). PTPN22 variation is associated with autoimmunity (4–6) and is the second most significant genetic risk factor for type 1 diabetes outside the HLA region (4,7). The PTPN22 1858T allele confers susceptibility to autoimmune diabetes because of an arginine to tryptophan substitution at position 620 (R620W) in the LYP protein. The R620W variation disrupts the association of LYP with Csk, a negative regulatory kinase (4), and a model was proposed in which this disruption causes increased phosphatase activity of LYP (8,9). It was suggested that R620W is a gain-of-function variant that dampens activation of T and B cells in response to antigen receptor ligation (3,8,10,11). Diminished signaling was indeed observed in both T and B cells from individuals carrying the 1858T allele (3,8,11). Several hypotheses have proposed how hyporeactivity may affect both T-cell (8) and B-cell (11,12) tolerance. Impaired T-cell receptor signaling could facilitate the escape of autoreactive T cells during thymic selection, impair the selection or activation of regulatory T (Treg) cells, or prevent the peripheral tolerization of autoreactive clones (8). Similarly, B cells may escape tolerance mechanisms by virtue of diminished activation and resistance to apoptosis in response to antigenic stimuli in individuals carrying the 1858T allele (11,12). Although these hypotheses provide a plausible explanation for the association of PTPN22 variation with autoimmunity, a recent publication reported data incompatible with this model (13). Using human samples and knockin mice carrying the homolog of the human LYP R620W variant (PEST domain-enriched tyrosine phosphatase [PEP] 619W in mouse), this study suggested that the disease variant protein was prone to rapid degradation, making PTPN22 1858T a loss-of-function allele. Results from these contradictory studies are difficult to reconcile. Therefore, whether LYP R620W is a gain- or loss-of-function variant remains controversial.

Characterization of Ptpn22-deficient C57BL/6 (B6) mice has provided more detailed insights into the role of this gene in lymphocyte function. In mice, Ptpn22 encodes the LYP homolog PEP. Loss of PEP was shown to facilitate positive selection but to have no impact on negative selection (14). The most notable effect observed in Ptpn22 knockout (KO) mice was the accumulation of effector/memory T cells (Teff) and their hyperreactivity compared with wild-type (WT) cells. In addition, Ptpn22 deficiency led to splenomegaly and lymphadenopathy in older mice but without causing autoimmune pathogenesis. A subsequent study showed that these mice also accumulated increased numbers of Treg cells (15), providing a possible explanation for the lack of overt autoimmunity in Ptpn22-deficient animals. Moreover, this same study demonstrated that Ptpn22 KO mice are less susceptible to the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (15). Although the original description of Ptpn22 KO mice suggested that the loss of this gene may predispose to autoimmunity, the latter report implies, rather, that loss of Ptpn22 may have an overall protective effect against autoimmune disease.

Because the LYP susceptibility variant R620W was reported to be gain of function (3,8–11), LYP inhibition was proposed as a potential therapeutic approach for type 1 diabetes (8,16). To investigate the function of PTPN22 in autoimmune diabetes and to evaluate whether inhibition of this gene may protect from disease, we generated transgenic NOD mice (nonobese diabetic) in which Ptpn22 can be silenced by RNA interference in a doxycycline-dependent manner. Gene silencing recapitulated phenotypic changes observed in Ptpn22 KO B6 mice. Ptpn22 knockdown (KD) did not, however, increase the risk of autoimmune diabetes in the NOD model, as would be predicted from the recent report claiming that LYP R620W...
is a loss-of-function variant. Instead, the findings support the notion that the susceptibility allele of PTPN22 is a gain-of-function variant.

**RESEARCH DESIGN AND METHODS**

**Mice.** All mice were bred and maintained under specific pathogen-free conditions at the University of Würzburg in accordance with institutional guidelines. Lentiviral transgenic P2 and P4 mice were generated as described previously (17). Briefly, lentivirus carrying an inducible short hairpin RNA (shRNA) expression cassette that targets the sequence AGTCTTGATGAGCAGGTTAT (P2) or AGGACTCTGCAGCACATTTTGA (P4) in the Ptpn22 mRNA was microinjected into NOD zygotes. For induction of gene silencing, mice were treated with 200 μg/mL doxycycline in the drinking water, unless otherwise noted. All experiments were approved by the Regional Government of Lower Franconia in accordance with German animal protection laws.

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RESULTS

Generation of inducible Ptpn22 KD NOD mice. To investigate the effect of PTPN22 gene variation within an experimental model of autoimmunity, we generated NOD mice in which Ptpn22 can be inducibly silenced. The NOD strain is a widely used model for type 1 diabetes (19), and the spontaneous onset of autoimmunity in this background makes it particularly useful to study genetic factors that contribute to disease risk. We used lentiviral transgenesis to introduce into NOD zygotes a construct encoding an shRNA against Ptpn22 under the control of a tetracycline-regulatable promoter (FH1t-UTG) (17,18). This lentiviral vector includes the coding sequences for the TetR and green fluorescent protein (GFP) (Fig. 1A). To control for possible off-target effects of RNA interference and for insertional effects of the transgene, we generated two transgenic lines with distinct shRNA sequences. We first validated the KD efficiency of several shRNA constructs against Ptpn22 using a luciferase reporter assay. After identifying two shRNA sequences that potently silenced the Ptpn22 reporter when expressed from a constitutively active promoter (Fig. 1B), we cloned these sequences, termed P2 and P4, into the inducible FH1t-UTG vector (18). We repeated the luciferase reporter assay with cells that had been transduced with the FH1t-P2-UTG or FH1t-P4-UTG lentivirus. We detected potent gene silencing only in cells grown in the presence of the inducing agent doxycycline (Fig. 1C), confirming the functionality of these inducible constructs. We proceeded with the generation of two transgenic lines using FH1t-P2-UTG and FH1t-P4-UTG lentivirus and obtained founders for both lines (Fig. 1D). These were bred for several generations to generate P2 and P4 mice carrying a single copy of the transgene (verified by Southern blotting of genomic DNA [data not shown]). Potent Ptpn22 KD was measured both at the mRNA and the protein level in doxycycline-treated but not in untreated transgenic mice (Fig. 2A and B). Of note, gene silencing was consistently more effective in P2 than in P4 mice (Fig. 2C).

Ptpn22 silencing expands the peripheral Treg-cell compartment. Transgenic mice were treated with doxycycline for various periods to investigate the effect of Ptpn22 silencing in adult animals. Although 10–14 days of treatment were sufficient to induce potent Ptpn22 KD (Fig. 2), we detected no change in the distribution or phenotype of immune cell populations after this short duration of treatment (data not shown). In contrast, prolonged treatment (>1 month) caused a significant increase in the proportion of FoxP3+ Treg cells in secondary lymphoid organs (Fig. 3A). To investigate whether the expansion of Treg cells in treated mice affected their function, we tested their activity in vitro and found that Ptpn22 KD Treg cells remained suppressive (Fig. 3B). These results are consistent with observations made in Ptpn22 KO B6 mice, where Treg cells are similarly increased and retain their suppressive function (15). The Treg-cell increase in secondary lymphoid organs of P2 and P4 mice appeared to be independent of thymic output because the proportion of FoxP3+CD4+CD8– thymocytes was not affected by gene silencing (Fig. 3C), even when Ptpn22 inhibition was initiated before birth (Fig. 3D). To further interrogate the origin of expanded Treg cells in Ptpn22 KD mice, we quantified the proportion of Helios-expressing FoxP3+CD4+ T cells in doxycycline-treated and -untreated mice (Fig. 3E). Helios was reported to be a marker for thymus-derived Treg cells, also known as natural Treg (nTreg) cells (20). Of note, subsequent data from several groups indicated that Helios could be detected in induced Treg (iTreg) cells in some instances (21–23), suggesting that this molecule may not be a reliable marker to differentiate nTreg from iTreg. Notwithstanding, because we found no change in the proportion of Helios+ Treg cells in the periphery of Ptpn22 KD mice, we interpret our data as signifying that no Treg subpopulation, whether Helios expressing or not, is preferentially expanded after Ptpn22 silencing. We also tested whether the loss of Ptpn22 affected the propensity of naïve T cells to convert into
FoxP3-expressing Treg cells as a thymus-independent explanation for the increase in Treg cells observed after gene silencing. However, naïve CD4+CD62Lhi T cells from WT and Ptpn22 KD mice did not differ in their potential for Treg conversion in vitro (Fig. 3F). Furthermore, we did not detect any difference in the acute proliferative behavior of Treg cells in vitro after Ptpn22 silencing (Fig. 3G and H). The data suggest that the entire peripheral Treg-cell compartment is expanded by loss of Ptpn22 and that neither thymic output of nTreg nor conversion of naïve T cells into iTreg cells is responsible for this expansion in Ptpn22 KD mice.
FIG. 4. Time of treatment determines the effect of Ptpn22 silencing on Teff-cell differentiation. A: The percentage CD44hiCD62Llo (Teff) cells within the CD4+ T-cell compartment were quantified in WT, P2, and P4 mice that had either been untreated, treated for 2 months from birth, or treated for 3 months from the age of 6 weeks. Data for CD8+ T cells were similar (not shown). Data are averaged (mean ± SEM) for two to six mice per group and are representative of two (doxycycline [dox] from birth) and six (dox from 6 weeks) similar experiments.

B: Cellularity of spleens from WT, P2, and P4 mice untreated or treated with dox for 4–5 months from the age of 10 weeks averaged (mean ± SEM) for 14 WT, 5 P2, and 8 P4 mice (untreated groups) and 19 WT, 10 P2, and 9 P4 mice (treated groups). C: Relative distribution of B cells, CD4 and CD8 T cells, dendritic cells (DC), and macrophages (Mp) in the spleen of WT and P2 mice either untreated or treated from the age of 4 weeks for a duration of 4 months. Data are averaged (mean ± SEM) from five mice per group and representative of four similar experiments. D: Quantification of naïve (CD44loCD62Lhi) and effector (CD44hiCD62Llo) T cells in spleens of WT and P2 mice untreated or treated with dox for 35 days. E: Quantification of the percentage of CD4+ and CD8+ T cells in the spleen of WT and P2 mice untreated or treated with anti-CD3 antibody.
**Time of treatment determines the effect of Ptpn22 silencing on T-cell differentiation.** The loss of Ptpn22 in gene-deficient B6 mice was shown to cause a significant expansion of Teff cells in older mice (14). In Ptpn22 KD mice, a similar increase in CD44hiCD62Llo T cells was apparent when gene silencing was induced from birth (Fig. 4A). In contrast, even though induction of Ptpn22 silencing in adult mice for a duration of several months caused significant splenomegaly (Fig. 4B), expansion of the T-cell compartment was almost undetectable after delayed gene silencing (Fig. 4A). The overall distribution of lymphocyte populations in the spleen of treated mice was not significantly altered, suggesting that homeostasis of both T and B cells was increased by Ptpn22 inhibition (Fig. 4C), although this effect was markedly weaker in the CD8+ T-cell compartment. Using two different strategies, we tested the propensity of naive T cells to differentiate into Teff cells more directly. First, we transferred a mixture of WT and transgenic CD4+ T cells into NOD.scid recipients and examined the fate of these cells 1 month after transfer. In this setting, T cells from Ptpn22 KD animals did not differ from WT cells in their propensity to differentiate into Teff cells (Fig. 4D). In contrast, when we injected WT or transgenic mice with anti-CD3 antibody, we found that Ptpn22 silencing greatly promoted the activation and differentiation of T cells after this stimulus (Fig. 4E). The difference between these two experiments may be accounted for by the fact that the acquisition of an effector phenotype following lymphopenia-induced expansion likely differs from that caused by a strong T-cell receptor stimulus, such as anti-CD3. The data thus confirm that Ptpn22 modulates the propensity of naive cells to differentiate into Teff cells but suggest that the increase of Teff cells observed in Ptpn22 KO mice largely depends on altered thymic selection in early stages of development when thymic output is most influential on the peripheral T-cell repertoire. Our inducible system demonstrates that Ptpn22 inhibition in adult animals has a lesser impact on the differentiation of naive T cells into Teff cells, at least under homeostatic conditions. This contrasts with effects on Treg cells that were detectable irrespective of the timing of Ptpn22 silencing.

**B-cell activation and apoptosis are increased by Ptpn22 silencing.** Characterization of Ptpn22 KO mice had revealed an increase in serum antibody titers (14), albeit no increase in autoantibodies and no detailed analysis of B-cell function have been reported from these mice to date. In humans, B cells from carriers of the PTPN22 susceptibility allele display decreased activation (3,10,11) and resistance to apoptosis (11). In addition, LYP R620W was found to reduce Syk, Akt, and PLCγ2 phosphorylation after B-cell receptor ligation (11), which would be consistent with a gain of function of this variant. In light of these observations, we sought to directly correlate loss of PEP activity with B-cell function using Ptpn22 KD mice. Ptpn22 silencing increased the upregulation of both CD25 and CD69 in B cells after anti-IgM stimulation (Fig. 5A). Furthermore, a higher fraction of B cells became CD25+CD69hi in response to both anti-IgM and anti-CD40 when Ptpn22 expression was inhibited (Fig. 5B). At the same time, Ptpn22 KD B cells displayed decreased survival in culture compared with WT cells (Fig. 5C). Although cells from P2 and P4 mice were concordant in most respects, the difference in KD efficiency between these two transgenic lines became most apparent when B-cell proliferation was quantified after in vitro stimulation (Fig. 5D). B cells from P2 mice proliferated more vigorously than cells from both WT and P4 animals in response to both anti-IgM and anti-CD40. Similarly, B cells from P2 mice displayed increased phosphorylation of PLCγ2, but Ptpn22 silencing in P4 cells was insufficient to effect a detectable change in PLCγ2 phosphorylation (Fig. 5E).

These combined results demonstrate that the inhibition of Ptpn22 has the exact opposite effect on B-cell signaling compared with the human R620W variant, which decreases activation and PLCγ2 phosphorylation and increases survival in response to stimulation (3,10,11).

**Ptpn22 silencing protects P2 mice from autoimmune diabetes.** Based on evidence that disease-associated LYP R620W is a gain-of-function variant, it was suggested that LYP inhibition may protect against autoimmunity (8). Even though the PTPN22 variation does not associate with multiple sclerosis, PEP deficiency was recently found to reduce the severity of EAE (15). The generation of an inducible Ptpn22 KD model was aimed at evaluating the therapeutic potential of Ptpn22 inhibition with a more clinically relevant approach. The absence of PEP throughout development in gene-deficient mice causes changes in thymic selection. The ensuing effects on the developing immune system likely differ from alterations in cell function caused by the acute inhibition of Ptpn22 in mature lymphocytes. We therefore opted to initiate Ptpn22 silencing in adult animals to better mimic a therapeutic intervention by LYP inhibition. To this end, we measured the frequency of diabetes in transgenic mice and their WT littermates that either had been left untreated or received doxycycline from the age of 10 weeks. Doxycycline-treated P2 but not P4 mice were significantly protected from diabetes compared with both untreated mice and treated WT mice (Fig. 6A and B). Disease frequency did not differ among untreated P2, P4, and WT mice. P4 mice showed no protection from disease, even when treated from an earlier time point of 4 weeks of age (Fig. 6C). We speculate that the weaker Ptpn22 KD in P4 mice may explain the difference in disease susceptibility between the two transgenic lines. This would be consistent with our observation that P4 lymphocytes showed less marked differences from the WT phenotype than P2 cells. Even though this difference in disease susceptibility may need to be resolved, it is apparent that Ptpn22 inhibition did not increase the risk of autoimmune diabetes in either transgenic line, contrary to what would be predicted from a study claiming that R620W is a loss-of-function variant (13). Instead, consistent with results in the EAE model (15), Ptpn22 silencing protected from autoimmune pathology.

**DISCUSSION**

After the discovery of the PTPN22 association with autoimmune diabetes, several groups carried out functional

and effector (CD44hiCD62Llo) cells in mixed WT and P2 or WT and P4 CD4+ T-cell populations before and after (32–35 days) transfer into NOD.scid mice. WT and transgenic cells were identified on the basis of GFP expression. E: The percentage of CD44hiCD62Llo (Teff) cells was quantified in CD4+ and CD8+ T cells from WT and P2 mice (pretreated with dox for 10 days) 2 days after injection with PBS only or anti-CD3 antibody 10 µg. Data in D and E are averaged from three to four mice per group and representative of two independent experiments each. *P < 0.05; **P < 0.01.
studies with lymphocytes from individuals who carry the susceptibility variant and found evidence that the disease allele is a gain-of-function variant (3,8–11). Data from animal models did not unequivocally support these findings because Ptpn22 KO mice appear to present signs of lymphocyte activity predisposing to autoimmunity (14). Furthermore, combining Ptpn22 deficiency with a mutant allele of CD45 caused lupus-like autoimmunity in double-mutant mice, and this evidence was interpreted as the disease allele being a loss-of-function variant (24). Finally, a notable study published in 2011 suggested that the PTPN22 disease variant encodes an LYP protein that is more prone to degradation than the protective variant. Knockin of a mutant Ptpn22 allele that encodes PEP 619W, the homolog of human LYP R620W, caused a phenotype reminiscent of Ptpn22 KO (13). However, despite an accumulation of Teff cells and increased serum antibody levels, Ptpn22 KO mice do not develop any signs of overt autoimmunity (14) possibly because of an increase in Treg-cell numbers (15). The present data from Ptpn22 KD NOD mice confirm that the peripheral Treg-cell compartment is increased in the absence of PEP. In contrast to Ptpn22 KO B6 mice, however, transgenic NOD mice did not have a higher proportion of FoxP3+ thymocytes. We observed in NOD mice that the expansion of Treg cells caused by the loss of PEP can occur independently of thymic output, indicating that Ptpn22 plays a role in the peripheral homeostasis of Treg cells. Of note, Treg-cell numbers in Ptpn22 KO mice were more substantially increased than in our KD model, and this may be due to both the incomplete loss of PEP in Ptpn22 KO mice and the additive effect in KO mice of greater thymic output together with direct effects on peripheral Treg cells. In this regard, however, it has been reported that a significant proportion of FoxP3+ cells found in the thymus is not recently generated but may in fact represent either long-term resident cells or cells that have recirculated into the thymus from the periphery (25,26). In light of the recent observation that the homeostasis of thymic resident Treg cells depends on signals distinct from those governing Treg-cell development (26), this raises the possibility that the increased Treg-cell population observed in the thymus of Ptpn22 KO mice (15) may in fact be a consequence of the expansion of the mature Treg-cell compartment.

How Ptpn22 silencing exerts its effect on peripheral Treg cells is not entirely clear, but we suggest that conversion of naïve T cells into iTreg cells is not involved. First, we did not observe increased efficiency of Treg conversion in vitro after Ptpn22 silencing, and second, the proportion of Helios-negative FoxP3+ cells [presumed thymic Treg cells (20), although some iTreg cells may also express Helios (21–23)] was not altered after Treg-cell expansion. Loss of PEP did not change the acute proliferative behavior of Treg cells but may instead increase homeostatic T-cell receptor signals essential for their peripheral maintenance and could decrease their requirement for IL-2 (27), allowing expansion of the Treg-cell niche. Alternatively, IL-2 levels may increase as a consequence of facilitated differentiation and activation of Teff cells and/or other immune cells producing IL-2, and loss of
Ptpn22 could thereby promote Treg-cell expansion in a cell-extrinsic manner. Although neither the present study nor the previous report demonstrating expansion of Treg cells in Ptpn22-deficient animals showed a direct link between this expansion and protection from autoimmunity, it is tempting to speculate a causal effect. Both protection from EAE in Ptpn22 KO mice and protection from autoimmune diabetes in P2 NOD mice shown here may be imparted by a more potent Treg-cell compartment.

Unlike its effect on Treg cells, Ptpn22 KD modulation of Teff-cell differentiation seemed to take effect primarily at the level of thymic development. Treatment of Ptpn22 KD mice from birth had a vastly more significant outcome than gene silencing initiated in adult mice. This may be explained by changes in positive selection described previously (14). Alterations of thymic selection can be predicted to have a lesser impact on the peripheral T-cell repertoire once thymic output starts waning (i.e., in adult animals).

Further, our work demonstrates that loss of Ptpn22 causes hyperreactivity and decreases survival of B cells. Because B cells are essential for the full penetrance of autoimmune diabetes in NOD mice (28), a defect in B-cell function may synergize with increased Treg-cell numbers in reducing the risk of autoimmune diabetes. In this context, it is of interest that the lack of protection observed in P4 mice correlates with a lesser dysfunction of B cells in vitro, and further work will be required to determine the relative contributions of the Treg- and B-cell compartments to the protected disease phenotype of P2 mice.

Data reported for B cells from individuals carrying the PTPN22 susceptibility allele are contradictory (10–13). The phenotype of Ptpn22 KD B cells presented here is the exact opposite of observations made for R620W by groups suggesting a gain of function (10,11) yet matches data obtained for this variant by the group proposing a loss of function (13). Consequently, we cannot draw an unequivocal conclusion about what functional change the human disease variant may cause based solely on cellular phenotypes. However, because we did not observe disease exacerbation after Ptpn22 inhibition, the findings are difficult to reconcile with the hypothesis that the PTPN22 disease allele is a loss-of-function variant caused by accelerated degradation of LYP (13). Instead, the results are most consistent with LYP R620W being a gain-of-function variant. In conclusion, we suggest that the present study of Ptpn22 inhibition in the NOD mouse model supports earlier claims that the susceptibility allele of PTPN22 is a gain-of-function variant and that inhibition of LYP may have therapeutic value for the treatment of autoimmunity.

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P.Z. designed and performed all experiments, analyzed data, and gave input on the manuscript. S.K. conceived and supervised the project, analyzed data, and wrote the manuscript. S.K. is the guarantor of this study and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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